

RESEARCH
Monograph-20

Initial
Sensitivity to
Alcohol



U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration

LIBRARY
ADDICTED
BALTIMORE CENTER

RESEARCH MONOGRAPH NO. 20

Initial Sensitivity to Alcohol

Proceedings of a Workshop on Alcohol Intoxication
October 13-14, 1988
Keystone, Colorado

Sponsored by:

National Institute on Alcohol Abuse and Alcoholism
and
University of Colorado Alcohol Research Center

Edited by:

Richard A. Deitrich, Ph.D., and
Albert A. Pawlowski, Ph.D.

NATIONAL INSTITUTES OF HEALTH
NIH LIBRARY
SEP 26 2007

BLDG 10, 10 CENTER DR
BETHESDA, MD 20892-1150

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration
National Institute on Alcohol Abuse and Alcoholism
5600 Fishers Lane
Rockville, Maryland 20857

The Editors: Richard A. Deitrich, Ph.D., is professor of pharmacology and vice chairman of the Department of Pharmacology at the University of Colorado Medical Center in Denver. He is also co-scientific director for the National Alcohol Research Center at the University of Colorado, supported under a grant from NIAAA. Prior to leaving NIAAA, Albert A. Pawlowski, Ph.D., was associate director, Division of Basic Research, and was responsible for administering the National Alcohol Research Centers Program. He is currently vice president of the Alcoholic Beverage Medical Research Foundation in Baltimore, Maryland.

The National Institute on Alcohol Abuse and Alcoholism has obtained permission from the copyright holders to reproduce figures as noted in the text. Further reproduction of these materials is prohibited without specific permission of the copyright holders. All other material contained in the monograph, except quoted passages from copyrighted sources, is in the public domain and may be reproduced without permission. Citation of the source is appreciated.

The U.S. Government does not endorse or favor any specific commercial product or commodity. Trade or proprietary names appearing in this publication are used only because they are considered essential in the context of the studies reported herein.

DHHS Publication No. (ADM) 90-1611

Printed 1990

Foreword

This monograph presents a record of the proceedings of a symposium, "Mechanisms of Initial Sensitivity of Ethanol," cosponsored by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) and the University of Colorado. More than 50 scientists from the United States and Finland participated through presentations of their research findings, contributions to roundtable panels, and general discussions of these new research discoveries.

The National Alcohol Research Center at the University of Colorado, located at the Medical Center in Denver and the main campus in Boulder, is 1 of 12 centers funded by NIAAA. Each center focuses on a central theme of importance to alcoholism, ranging from the neurophysiologic effects of alcohol consumption to the study of factors influencing drinking patterns and trends and the prevention of alcohol problems. In addition to their primary mission of developing new knowledge by conducting original research in their chosen areas of expertise within the field of alcoholism, the centers have the further responsibility of disseminating this knowledge to the scientific and lay communities. In this connection, the centers have joined NIAAA in organizing and holding workshops and conferences on topics of immediate interest to their specialty areas. The proceedings reported in this volume represent another in the series of such collaborative activities.

The purpose of the symposium, which was held Oct. 13-14, 1988, in Keystone, CO, was to provide a state-of-the-art review of central nervous system mechanisms underlying the brain's sensitivity to the effects of alcohol as a pharmacologic agent. The symposium consisted of 22 presentations grouped under 4 major headings: (1) Selective Breeding for Initial Sensitivity, (2) Cell Membranes: Structure and Function, (3) Neuropeptides, and (4) GABA-Benzodiazepine Receptors: Calcium Channels. In addition, discussions of these reports led to recommendations for future research.

It is hoped that these proceedings will stimulate neuroscientists to think about the effects of alcohol on the brain and mechanisms underlying alcohol intoxication. Although this monograph presents highly technical material, it is presented with the confidence that the research findings will relate importantly to a broader understanding of alcohol's effects on the brain.

Albert A. Pawlowski
Associate Director
Division of Basic Research
National Research Centers Program
National Institute on Alcohol Abuse
and Alcoholism

Preface

This monograph came about as a result of a conference held in Keystone, CO in October 1988. The topic was the mechanism of the initial reactions to ethanol. In some respects, this is an offspring of a conference held 10 years earlier entitled "Development of Animal Models as Pharmacogenetic Tools." That conference and monograph laid the foundation for many of the genetic studies reported on in this monograph. In addition, many other tools for the investigation of acute effects of ethanol have been used and are reported on herein.

There are a great many effects of ethanol, but it is the ataxic, incoordinating effects that lead to many of the serious consequences such as automobile and industrial accidents, suicide, and social and legal problems. Progress in understanding the molecular and cellular mechanisms of these effects has been explosive in the past few years compared with the decades of relative lack of advancement of knowledge beyond the simple statement that ethanol acts like all other general anesthetics. Of course this was cold comfort, since we did not know how general anesthetics acted either. Modern methods of physical chemistry, electrophysiology, biochemistry, and molecular biology have allowed us to see more clearly the possibilities of the molecular actions of ethanol. It is axiomatic among pharmacologists that once the action of a compound is understood, it may then be possible to design chemicals or procedures to interfere with the initial actions of the compound or the consequences of its effects. We are now much closer to realizing that goal for ethanol than would have been thought possible 10 years ago. If the next 10 years bring about as much advance as have the past 10, the next conference on initial effects of ethanol should be exciting indeed.

Richard A. Deitrich
Department of Pharmacology
and
Alcohol Research Center
University of Colorado
School of Medicine

Participants

Ronald L. Alkana
Alcohol and Brain Research
Laboratory
School of Pharmacy
University of Southern California
Los Angeles, CA 90033

Andrea M. Allan
Department of Pharmacology
University of Colorado School of
Medicine
4200 E. 9th Avenue, C236
Denver, CO 80262

Rodney C. Baker
Department of Pharmacology
University of Colorado School of
Medicine
Denver, CO 80262

George R. Breese
Center for Alcohol Studies and
Biological Sciences Research
Center
School of Medicine
University of North Carolina
Chapel Hill, NC 27599

Karen T. Britton
Department of Basic and Clinical
Research
Scripps Clinic and Research
Foundation
10666 North Torrey Pines Road
La Jolla, CA 92037

M. Bureau
Department of Pharmacology
School of Medicine
University of California, Los
Angeles
Los Angeles, CA 90024

Scott Cain
Department of Psychiatry &
Pharmacology
Duke University Medical Center
Durham, NC 27710

Allan Collins
Institute for Behavioral Genetics
Box 447
University of Colorado
Boulder, CO 80309

Initial Sensitivity to Alcohol

John C. Crabbe
Research Service (151P)
Veterans Administration Medical
Center
Portland, OR 97201

Hugh E. Criswell
Center for Alcohol Studies and
Biological Sciences
Research Center
School of Medicine
University of North Carolina
Chapel Hill, NC 27599

Richard A. Deitrich
Department of Pharmacology
University of Colorado School of
Medicine
4200 East 9th Avenue, C236
Denver, CO 80262

L. Deng
Department of Pharmacology
School of Medicine
University of California, Los
Angeles
Los Angeles, CA 90024

Cindy L. Ehlers
Department of Basic and Clinical
Research
Scripps Clinic and Research
Foundation
10666 North Torrey Pines Road
La Jolla, CA 92037

Carl Erickson
Drug Dynamics Institute
School of Pharmacy
University of Texas
Austin, TX 78712

C.J. Peter Eriksson
Research Laboratories of ALKO,
Ltd.
POB 350
SF-00101 Helsinki, Finland

V. Gene Erwin
Alcohol Research Center
School of Pharmacy
Box 297
University of Colorado
Boulder, CO 80309

Daniel J. Feller
Research Service
Veterans Administration Medical
Center
Portland, OR 97201

Dora B. Goldstein
Department of Pharmacology
Stanford University School of
Medicine
Stanford, CA 94305

Enoch Gordis
National Institute on Alcohol
Abuse and Alcoholism
5600 Fishers Lane
Room 16-105
Rockville, MD 20857

Participants

N. Harris
Department of Pharmacology and
Department of Psychiatry and
Human Behavior
Thomas Jefferson University
Jefferson Medical College
Philadelphia, PA 19107

R. Adron Harris
Department of Pharmacology
University of Colorado Health
Sciences Center
4200 East 9th Avenue, C236
Denver, CO 80262

Barry Hoffer
Department of Pharmacology
University of Colorado School of
Medicine
4200 East 9th Avenue, C236
Denver, CO 80262

Paula L. Hoffman
Division of Intramural Clinical
and Biological Research
National Institute on Alcohol
Abuse and Alcoholism
12501 Washington Avenue
Rockville, MD 20852

K.-Y. Huh
Department of Pharmacology
School of Medicine
University of California, Los
Angeles
Los Angeles, CA 90024

Walter A. Hunt
Behavioral Sciences Department
Armed Forces Radiobiology
Research Institute
Bethesda, MD 20814-5145

Wendy Keir
Department of Pharmacology
School of Medicine
University of Colorado Health
Sciences Center
4200 East 9th Avenue, C236
Denver, CO 80262

George F. Koob
Department of Basic and Clinical
Research
Scripps Clinic and Research
Foundation
10666 North Torrey Pines Road
La Jolla, CA 92037

S.K. Kulkarni
Department of Pharmacology
University of Texas Health
Science Center at San Antonio
7703 Floyd Curl Drive
San Antonio, TX 78284-7764

Nancy M. Lee
Department of Pharmacology
University of California Medical
Center
San Francisco, CA 94122

Initial Sensitivity to Alcohol

Steven Leslie
College of Pharmacy
University of Texas at Austin
Austin, TX 78712

T.-K. Li
Emerson Hall 421
Indiana University School of
Medicine
Indianapolis, IN 46223

Horace H. Loh
Department of Pharmacology
University of California Medical
Center
San Francisco, CA 94122

S.G. Madamba
Alcohol Research Center
Research Institute of Scripps
Clinic
10666 North Torrey Pines Road
La Jolla, CA 92037

R.J. Marley
Department of Psychiatry
Yale University
New Haven, CT 06520

Gerald McClearn
College of Human Development
The Pennsylvania State University
University Park, PA 16802

Todd D. McIntyre
Laboratory of Neuroscience
National Institute of Diabetes,
Digestive and Kidney Diseases

National Institutes of Health
Building 8, Room 105
Bethesda, MD 20892

Barbara J. Martin
Institute for Behavioral Genetics
University of Colorado
Boulder, CO 80309

A.K. Mhatre
Department of Pharmacology
University of Texas Health
Science Center
7703 Floyd Curl Drive
San Antonio, TX 78284-7764

S.D. Moore
Alcohol Research Center
Research Institute of Scripps
Clinic
10666 North Torrey Pines Road
La Jolla, CA 92037

Richard W. Olsen
Department of Pharmacology
School of Medicine
University of California, Los
Angeles
CJS 23-278
Los Angeles, CA 90024

Michael R. Palmer
Department of Pharmacology,
C236
University of Colorado Health
Sciences Center
4200 East 9th Avenue
Denver, CO 80262

Participants

Albert A. Pawlowski
Division of Basic Research
National Institute on Alcohol
Abuse and Alcoholism
Room 14-C-20
5600 Fishers Lane
Rockville, MD 20857

Tamara J. Phillips
Research Service
Veterans Administration Medical
Center
Portland, OR 97201

June I. Pounder
Institute for Behavioral Genetics
University of Colorado
Boulder, CO 80309

George R. Siggins
Alcohol Research Center
Research Institute of Scripps
Clinic
10666 North Torrey Pines Road
La Jolla, CA 92037

James M. Sikela
Department of Pharmacology
School of Medicine
University of Colorado Health
Sciences Center
4200 East 9th Avenue, C236
Denver, CO 80262

Phil Skolnick
Laboratory of Neuroscience
National Institute of Diabetes,

Digestive and Kidney Diseases
National Institutes of Health
Building 8, Room 103
Bethesda, MD 20892

Andrew P. Smith
Department of Pharmacology
University of California Medical
Center
San Francisco, CA 94122

G. Smith
Department of Pharmacology
School of Medicine
University of California, Los
Angeles
Los Angeles, CA 90024

Karen Spuhler
Department of Pharmacology
University of Colorado School of
Medicine
Denver, CO 80262

Boris Tabakoff
Division of Intramural Clinical
and Biological Research
National Institute on Alcohol
Abuse and Alcoholism
National Institutes of Health
Clinical Center
Building 10, Room 3C103
Bethesda, MD 20892

Initial Sensitivity to Alcohol

J. Taylor
Department of Pharmacology and
Department of Psychiatry and
Human Behavior
Thomas Jefferson University
Jefferson Medical College
Philadelphia, PA 19107

Maharaj K. Ticku
Department of Pharmacology
University of Texas Health
Science Center at San Antonio
7703 Floyd Curl Drive
San Antonio, TX 78284

Ramon Trullas
Laboratory of Neuroscience
National Institute of Diabetes,
Digestive and Kidney Diseases
National Institutes of Health
Building 8, Room 117
Bethesda, MD 20892

Wolfgang H. Vogel
Department of Pharmacology and
Department of Psychiatry and
Human Behavior
Thomas Jefferson University
Jefferson Medical College
Philadelphia, PA 19107

Jeanne M. Wehner
Institute for Behavioral Genetics
Box 447
University of Colorado
Boulder, CO 80309

Donna Wilson-Shaw
Department of Pharmacology
School of Medicine
University of Colorado
Denver, CO 80262

Contents

Foreword	iii
Preface	v
Participants	vii

Selective Breeding for Initial Sensitivity

Selective Breeding for Initial Sensitivity: Introduction, <i>Ting-Kai Li</i>	3
---	---

Selective Breeding of Mice and Rats for Initial Sensitivity to Ethanol: Contributions to Understanding of Ethanol's Actions, <i>Richard A. Deitrich</i>	7
---	---

Finnish Selective Breeding Studies for Initial Sensitivity to Ethanol: Update 1988 on the AT and ANT Rat Lines, <i>C. J. Peter Eriksson</i>	61
---	----

Selective Breeding of Rats Differing in Sensitivity to the Hypnotic Effects of Acute Ethanol Administration, <i>Karen Spuhler, Richard A. Deitrich, and Rodney C. Baker</i>	87
---	----

Initial Sensitivity to Ethanol

Alcohol-Stress Interaction: A Genetically Determined Relationship? <i>Wolfgang H. Vogel, N. Harris, and J. Taylor</i>	103
---	-----

Selective Breeding for Two Measures of Sensitivity to Ethanol, <i>John C. Crabbe, Daniel J. Feller, and Tamara J. Phillips</i>	123
--	-----

Cell Membranes: Structure and Function

Cell Membrane Fluidity as a Determinant of Intoxication: Status of the Hypothesis, <i>Dora B. Goldstein</i>	155
---	-----

Discussion: Cell Membrane Fluidity as a Determinant of Intoxication: Status of the Hypothesis, <i>Ronald L. Alkana</i>	171
--	-----

Ethanol and the Sodium Channel, <i>Walter A. Hunt</i>	185
---	-----

Electrophysiological Evaluation of Acute Ethanol Effects on Transmitter Responses in Central Neurons, <i>George R. Siggins, S. G. Madamba, and S. D. Moore</i>	197
--	-----

Neuropeptides

Evidence for Site Selectivity of Thyrotropin-Releasing Hormone Interactions With Ethanol-Induced Central Nervous System Dysfunction, <i>Hugh E. Criswell and George R. Breese</i>	219
---	-----

Ethanol and Opiate Receptors, <i>Paula L. Hoffman and Boris Tabakoff</i>	233
--	-----

Purification, cDNA Cloning, and Characterization of an Opioid-Binding Protein with Homologies to the Immunoglobulin Superfamily, <i>Andrew P. Smith, Horace H. Loh, and Nancy M. Lee</i>	245
--	-----

Corticotropin-Releasing Factor, Stress, and Ethanol, <i>George F. Koob, Karen T. Britton and Cindy L. Ehlers</i>	263
--	-----

Contents

Neurotensinergic Processes: Potential Role In Ethanol Actions, <i>V. Gene Erwin</i>	279
---	-----

GABA-Benzodiazepine Receptors: Chloride Channels

Structure of the γ -Aminobutyric Acid-Benzodiazepine Receptor Protein Complex: Focus on Central Nervous System Depressant Drug Action, <i>R. W. Olsen, L. Deng, K.-Y. Huh, G. Smith, and M. Bureau</i>	303
---	-----

Molecular Genetic Study of the Role of the γ -Aminobutyric Acid-Benzodiazepine Receptor in Neurosensitivity to Alcohol, <i>James M. Sikela, W. Keir, and D. Wilson-Shaw</i>	317
--	-----

Brain Chloride Channels and Alcohol Action: Neurochemical Studies, <i>R. Adron Harris and Andrea M. Allan</i>	323
---	-----

Behavioral Studies of GABAergic responses in LS and SS mice: Are Ethanol Sensitivity and Responses to GABAergic Agents Regulated by Common Mechanisms? <i>Jeanne M. Wehner, Barbara J. Martin, R. J. Marley, and June I. Pounder</i>	345
--	-----

Does the Benzodiazepine- γ -Aminobutyric Acid Receptor-Chloride Ionophore Complex Mediate Initial Sensitivity to Ethanol?: Studies Using Long-Sleep (LS) and Short-Sleep (SS) Mice, <i>Todd D. McIntyre, Ramon Trullas, and Phil Skolnick</i>	381
--	-----

Ethanol and GABAergic Transmission, <i>Maharaj K. Ticku, A. K. Mehta, M. Mhatre, and S. K. Kulkarni</i>	401
---	-----

Electrophysiologic Interactions of Ethanol with Ro 15-4513 and γ -Aminobutyric Acid Mechanisms on Rat and Human Neurons, <i>Michael R. Palmer</i>	419
--	-----

Selective Breeding for Initial Sensitivity

Selective Breeding for Initial Sensitivity: Introduction

*Ting-Kai Li*¹

The topic of this opening session is selective breeding for genetic differences in the initial response to ethanol. Before launching into descriptions of the many excellent animal models that have been developed to study potential mechanisms of central nervous system sensitivity to ethanol, we should first establish what is generally meant by initial sensitivity. Most investigators would agree that, broadly defined, the term denotes the response of an animal upon first exposure to a single dose of ethanol. This response has been assessed by a variety of behavioral and physiologic measures, and the degree to which differences are observed within a population appear to be not only species specific but also test specific.

Because of the phenomenon of acute tolerance (Erwin et al. 1980), it is important to distinguish tests that specifically measure a change in function from those that measure recovery of function. Acute or within-session tolerance is tolerance occurring within the time course of a single session of testing. For example, "sleep time" is actually the time required for an animal to regain the righting reflex. A difference in sleep time may derive from an innate difference in the brain's sensitivity to the anesthetic action of ethanol, a difference in development of acute tolerance, or both. In investigating mechanisms at the cellular or molecular level, we need to be aware that even within a short period of exposure, neuroadaptive changes can occur.

¹Indiana University School of Medicine, Indianapolis, IN 46223

Selective Breeding for Initial Sensitivity

Other important variables that warrant attention are dose and route of administration. Absorption characteristics and blood-brain alcohol concentration profiles can be quite different with different routes of administration. Ethanol's actions are biphasic, and they are generally thought to be rewarding at low concentrations but aversive at moderate to high concentrations. Most programs of selective breeding for differences in sensitivity to ethanol have used doses that impaired function (e.g., studies with LS/SS mice, MA/LA rats, AT/ANT rats, HAS/LAS rats, and HOT/COLD mice). Whether FAST/SLOW mice, selected on the basis of differences in ethanol-stimulated open-field activity with relatively low doses of ethanol, represent response differences to the rewarding or "euphorogenic" effects of ethanol is not yet known. There is clear evidence that animals can develop tolerance to the aversive effects of ethanol, but it is still uncertain whether tolerance (or reverse tolerance) occurs with the low-dose effects.

Studies in the selectively bred alcohol-preferring P line of rats have shown that oral consumption of ethanol solutions in concentrations as high as 30 percent (vol/vol) is reinforcing. By contrast, ethanol solutions higher than 5 percent become aversive to the selectively bred alcohol-nonpreferring NP line (Froehlich et al. in press). Conditioned taste aversion experiments have also shown that ethanol administered intraperitoneally is aversive to NP rats at lower doses than in P rats (Gatto et al. 1987). Moreover, P rats develop acute tolerance to moderate-high doses of ethanol more rapidly than do NP rats, and they appear able to maintain this tolerance for a longer period of time (Li et al. 1988; Tabakoff et al. 1982). Indeed, one of the strongest associations of ethanol preference appears to be development of acute tolerance (Li et al. 1988; Waller et al. 1983). Thus, both the reinforcing properties of ethanol and the capacity of the animal to develop tolerance are important determinants of alcohol consumption behavior. In looking at any mechanisms of ethanol sensitivity, whether it be the monoaminergic dopamine and serotonin systems, neuropeptides, or the γ -aminobutyric acid (GABA)-benzodiazepine receptor complex, we should not lose sight of how the mechanism relates to alcohol-seeking behavior, which is at the core of the disorder alcoholism.

References

- Erwin, V.G.; McClearn, G.E.; and Kuse, A.R. Interrelationships of alcohol consumption, actions of alcohol and biochemical traits. *Pharmacol Biochem Behav* 13 (Suppl. 1):297-302, 1980.
- Froehlich, J.C.; Harts, J.; Lumeng, L.; and Li, T.-K. Differences in response to the aversive properties of ethanol in rats selectively bred for oral ethanol preference. *Pharmacol Biochem Behav* (in press).
- Gatto, G.J.; Murphy, J.M.; Waller, M.B.; McBride, W.J.; Lumeng, L.; and Li, T.-K. Persistence of tolerance to a single dose of ethanol in the selectively bred alcohol-preferring rats. *Pharmacol Biochem Behav* 28:105-110, 1987.
- Li, T.-K.; Lumeng, L.; Froehlich, J.C.; Murphy, J.M.; and McBride, W.J. Genetic influence on response to the reinforcing properties of ethanol in rats. *Excerpta Med Int Congr Ser* 805:487-490, 1988.
- Tabakoff, B.; Melchior, C.L.; and Hoffman, P.L. Commentary on ethanol tolerance. *Alcoholism Clin Exp Res* 6:252-259, 1982.
- Waller, M.B.; McBride, W.J.; Lumeng, L.; and Li, T.-K. Initial sensitivity and acute tolerance to ethanol in the P and NP lines of rats. *Pharmacol Biochem Behav* 19:683-686, 1983.

Selective Breeding of Mice and Rats for Initial Sensitivity to Ethanol: Contributions to Understanding of Ethanol's Actions

*Richard A. Deitrich*¹

Introduction

The use of bidirectional selectively bred animals to obtain basic biologic information is a relatively recent endeavor for scientists even though this technique has been used in agriculture, at least in one direction, for thousands of years. The usefulness of selective breeding in science has received a great deal of attention in research on the effects of alcohol, perhaps because the tools for such studies have been difficult to obtain and the tedious work of selective breeding promises to produce new and better animal models. This paper is a review of one of the early programs initiated by McClearn and Kakihana (1981) in the 1960s to selectively breed for the acute anesthetizing effects of ethanol. Also included are some references to a replication of the selection in rats.

¹Department of Pharmacology and Alcohol Research Center, University of Colorado School of Medicine, Denver, CO 80231

Development of Heterogeneous Stock

Essential to any selective breeding program is the availability of a genetically heterogeneous group of animals from which selection is initiated. This is best accomplished by intercrossing a number of inbred strains of animals that have as little ancestral relationship to one another as possible and also have a wide range of responses for which one will eventually select. The advantage of using inbred strains to generate such a heterogeneous line is that the line can, at least in theory, be reconstructed at any time in the future. This approach also allows one to put into the base stock those behaviors upon which one wishes to apply selection pressure. In the case of the heterogeneous stock (HS) mice, eight inbred strains were reciprocally crossed; that is, both males and females from each strain were used in the crosses. This process yielded four F_1 crosses. Again the four F_1 stocks were crossed, using both males and females from each. This resulted in two F_2 lines, which were then crossed to yield the F_3 generation, from which selection could begin. It is essential that there be a large number of families from which to select. In the case of the HS mice, the inbred strains used were A, AK, BALB/c, C3H, DBA/2, Is/Bi, C57, and RIII. Forty breeding pairs are maintained without mating cousins and have been used to initiate a number of other selection studies.

History of Selection

The original rationale for developing these selected lines of animals was to provide a better tool for study of the initial actions of ethanol. Simultaneously, however, this strategy provided solid evidence that there is a genetic effect in the responses of mice to ethanol as well as information about the mechanism of action of other central nervous system (CNS) depressants. The following discussion not only details the history of selection but also summarizes the genetic influence on behavioral reactions to ethanol as indicated by studies of these animals.

For the first selected generation, McClearn and Kakahana (1981) used 44 males and 53 females to test for "sleep time" to a 3.3- g/kg dose of ethanol as a 30 percent (vol/vol) solution given intraperitoneally (IP). There were usually 10 breeding pairs in each line, and mass selection was used. That is,

the best 10 males and best 10 females were mated in both the short-sleep (SS) and long-sleep (LS) lines, provided they had no common grandparents. McClearn and Kakihana (1981) describe procedures used initially in the selection. The definition of sleep time by subsequent investigators has changed, primarily with respect to whether sleep time is taken from the time of injection or from the time that the animal cannot either hang on a wire mesh or right itself when placed in a trough. In practice, although there are small differences in the fall time of the selected mice after IP injection, sleep time is usually long enough that these differences are of no practical consideration. The other difference between the work of McClearn and Kakihana and later studies is in the definition of regain of the righting reflex. The term as normally used denotes the ability of the animal to right itself two (or three) times in 1 min (or 30 s). Again, slight variations are not crucial as long as the procedure is consistent. For many generations of selection, the blood ethanol level upon awakening was not obtained. This is now a routine procedure for most experiments with the animals, since when the blood is obtained from the retro-orbital sinus, these values give a clearer picture of brain sensitivity, uncontaminated by metabolic differences imposed by the selection process, the environment, or the experimental protocol. Numerous studies have shown that blood and brain ethanol do not reach a steady state for a number of minutes after the IP injection of ethanol (Smolen and Smolen submitted). Blood ethanol values obtained from the tail blood lag far behind the brain levels of ethanol and are not as useful as values for blood from the retro-orbital sinus. In any case, blood ethanol values obtained at sleep time values of less than 15-20 min are viewed with considerable suspicion.

The original selection was made on the basis of sleep time, and that procedure continued until selection was terminated, even though blood ethanol values were available in later generations.

There was a serious bottleneck in the LS line and, to a lesser extent, in the SS line. For generations 6 and 7 of the LS line, only a single breeding pair was available. The SS line was down to three breeding pairs at generation 7. This bottleneck has posed at least theoretical problems for all subsequent investigators, since it increases the inbreeding coefficient considerably. At generation 33 (generation 25 of selection), the inbreeding coefficients were at least 0.79 for the LS mice and 0.60 for the short-sleep mice (calculation by J.

DeFries, quoted in Goldman et al. 1985). While inbreeding is inevitable in all finite breeding populations, more recent selective breeding programs have used within-family selection so that each family is represented in subsequent generations. These studies have also used a rotation breeding program to minimize breeding of close relatives.

The other problem with this early selection study was that only a single high line (LS) and a single low line (SS) were started. In addition, the control line was that of the HS stock, but these animals were not tested for sleep time and thus make a less than ideal control line. Nevertheless, these animals have been extraordinarily useful in the field.

The selection dose was increased to 3.5 g/kg in generation 8 and to 4.2 g/kg in generation 9. It remained at this level until generation 25 (selected generation 17) when the dose was reduced to 3.8 g/kg for LS mice and increased to 4.7 g/kg for SS mice. Currently, it is not uncommon to use doses of 5 or 5.2 g/kg for experiments with SS mice and 2.8 or 3 g/kg for those with LS mice and achieve approximately the same sleep times. (Also see the paper by Smolen and Smolen [1987] that describes a threshold effect of ethanol for regain of the righting response and another by Smolen et al. [1987] on sensitivity of inbred as well as SS and LS mice.)

An interesting demonstration of the persistence of the differential sleep time response despite drastic environmental change is in a study by Erwin and McClearn (1981). They carried ova transplants from LS mice into foster SS mothers. The sleep time and blood ethanol values at regaining the righting response were not different from those for mice born and reared by LS mothers.

Selection was suspended at generations 6 and 7 because of mouse infertility and again at generations 19-24 because of infertility in the grant-funding process. Finally, selection was suspended altogether at generation 33, or after 25 generations of selection. (Nearly all investigators report the generation number, not the generations of selection.) At this time, a number of genetic markers showed that the mice were homozygous at several loci (Holmes et al. 1986) and in several brain polypeptide variants (Goldman et al. 1985). There are nevertheless still three color variants in the SS mice,

although albino predominates, and two color variants in the LS lines on which albino never occurs.

Calculations of heritability showed a value of 0.18 for both lines over five generations (McClearn and Kakihana 1981). Dudek and Abbott (1983) estimated 0.30. DeFries et al. (1988) estimated 0.4 from recombinant inbred data, and Spuhler et al. (1982) estimated 0.53.

Calculations of the number of genes involved have yielded markedly different results, depending on the method and investigator. Dudek and Abbott (1983) calculated 9 ± 2 genes from analysis of a Mendelian cross of SS and LS mice. These mice were from a stock maintained at the State University of New York (SUNY)-Binghamton by John Fuller and later moved to SUNY-Albany. They were obtained from the Institute for Behavioral Genetics at generation 18 and maintained without selection. On the other hand, Howerton et al. (1984a) have estimated that there are 3.32 genes involved in the sleep time response to ethanol in these mice. Certainly the distribution of sleep time values in the LSxSS recombinant inbred (RI) strains of mice indicates that several genes are involved, and the estimate based on these data is 7.1 (DeFries et al. submitted).

The foundation stock (the HS mice), in addition to being useful for initiating other selection studies, have been used to study several aspects of the action of ethanol. In one way, they are ideal subjects since they have been maintained in a large breeding colony and have undergone only normal random selection pressure from the environment. It is necessary to be able to carry out two measures on a single animal (sleep time and some biochemical parameter, for example) and then correlate the values from a large number of animals (Erwin et al. 1980). A second, related approach is to test a large group of HS mice for sleep time and then divide them arbitrarily into SS-like and LS-like groups. This procedure, which replicates the process that was carried out in the original selection study, has been done in at least three cases. The first was the study by Goldstein et al. (1982) on cell membrane-fluidizing effects of ethanol. Another was a study by Baker et al. (1987) in a study of 50 percent effective dose (ED_{50}) values for ethanol in these lines, and a third was a study of the effects of pentobarbital in these mice (Allan and Harris 1989). The results of these studies will be discussed in later sections.

Inbreeding and Recombinant Inbreeding

More recently, the SS and LS mice have been inbred and are available to investigators to establish their own colonies. These lines are designated ISS and ILS to avoid confusion with the outbred SS and LS lines that have been used until now. They have essentially the same sleep times, blood ethanol upon awakening, and temperature effects of ethanol as do the SS and LS lines (V.G. Erwin and J.C. DeFries personal communication).

The outbred SS and LS mice have also been used to generate 27 RI strains (DeFries et al. submitted). This breeding study was started with LS and SS mice that were not completely inbred at all loci. The rationale was that they certainly should be homozygous for all loci relevant to the ataxic effects of ethanol after 25 generations of selection. The other consideration was one of time that it would have required to completely inbreed the SS and LS mice and then undertake recombinant inbreeding.

In reviewing many of the correlated responses detected in these selected lines, I will make reference to several levels of confidence in these reports. The classification used is as follows:

- **Negative**—There is no correlation between the selected response (sleep time) and the measured trait. These are the strongest data that we can obtain, but they are useful only in directing subsequent research along a new path or to disprove some hypothesis.
- **Positive**—There is a significant correlation between the selected response and the trait measured. This correlation has been confirmed by study of strains, lines, or species besides the SS and LS mice. Although not proving a cause-effect relationship, findings in this category provide a solid basis for further investigation.
- **Probable**—This category is similar to the positive category, but the correlation has not been confirmed in other models. A great deal of the evidence that has been generated unfortunately falls into this category, but that situation is slowly changing as investigators realize that a difference between selected lines is only the beginning of an investigation and not an end in itself.

- **False-Positive**—A significant correlation between sleep time and a measured trait in the SS and LS mice cannot be confirmed in some other model. Now that selection studies are routinely carried out in replicate lines, it is possible to carry out the confirmation or lack of confirmation immediately. The problem is that if one has a significant correlation in one model but cannot confirm it in another, which is the correct observation? It may be only a problem of sensitivity of the assay or some other anomaly. Nevertheless, such a finding has thrown cold water on a number of the investigations reviewed here.

Metabolism of Ethanol

The metabolism of ethanol was the first biochemically correlated measure to be determined in SS and LS mice. Obviously, one hoped that the selection process had selected for a different CNS and not for a different liver. This has proven to be the case. The original study by Heston et al. (1974) found that there was no difference in the rate of disappearance of ethanol in generation 14 SS and LS mice given (presumably, although it is not stated in the paper) 4.1 g/kg of ethanol IP. Kakihana (1976a) also determined that the rates of ethanol disappearance did not differ between the two lines after a dose of 2 g/kg. She used mice of the 16th generation.

Since these early studies, much more detailed research on the factors influencing ethanol elimination rates has been carried out. Gilliam and Collins (1982a) reported that SS mice had a higher rate of clearance of ethanol than did LS mice at doses of both 2.5 and 4.1 g/kg. They also reported a circadian effect on ethanol metabolism. Howerton et al. (1983b) also reported a greater clearance of ethanol in SS mice after a dose of 4.1 g/kg. Gilliam et al. (1983a) found again that SS mice had a higher clearance rate. The apparent volume of distribution was also different, but it was dependent on the dose of ethanol given. They also found that the apparent K_m for ethanol disappearance was markedly different. SS mice had an apparent K_m of 425 mg/dl, whereas LS mice had a value of 110 mg/dl. Gilliam and Collins (1983b) also found that the metabolism, sleep time, and temperature effects of ethanol depended on the concentration of ethanol, which was given IP. Gilliam et al. (1985) compared the effects of ethanol when doses were given orally and IP. Later, Romm and Collins (1987)

demonstrated that the ethanol metabolic rate was directly correlated with the temperature of the animal. Since LS animals have a greater temperature drop than do SS animals at the same dose, it seems reasonable to suggest that the differences in metabolism may be only secondary to the selection for increased temperature differential between the lines. The suggestion in these papers that the differences in metabolism may have been imposed by the change in selection doses seems reasonable. However, there are two papers that directly address this question. Using mice from the Binghamton-Albany (B-A) colony, Phillips et al. (1984) also found that although there were some differences in clearance rate, these could not account for the sleep time differences. This is an important finding, since these mice had not undergone any selection pressure from generation 18. A study by Smolen et al. (1986) used mice that had undergone the full 25 generations of selection (generation 39 mice), and they gave a dose of 3.8 g/kg in a 20 percent (wt/vol) solution. A second study, by Smolen and Smolen (submitted), used a dose of 4 g/kg in a 20 percent (wt/vol) solution. The data from these three studies are presented in table 1.

Table 1.—Summary of three metabolism studies

Study	<u>Clearance (mg/dl/hr)</u>		<u>Vd (ml/g)</u>	
	LS	SS	LS	SS
Phillips et al. (1984)	58.2±2.0	75.5±3.6	0.88±0.02	0.88±0.02
Smolen et al. (1986)	69.6±2.4	82.8±1.2	0.77±0.01	0.79±0.01
Smolen and Smolen (Submitted)	70.2±2.5	84.1±6.8	0.85±0.02	0.83±0.03

It is always dangerous to compare results from two different laboratories or even from the same laboratory at different times. However, table 1 shows that LS mice that had not undergone the selection pressure had the lowest clearance rate (Phillips et al. 1984). LS mice that had undergone selection

pressure but at a decreased ethanol selection dose actually had a greater difference in clearance rates than did SS mice that had an increased selection dose imposed (58-70 mg/dl/hr for LS mice versus 75-83 mg/dl/hr for SS mice [Smolen et al. 1986; Smolen and Smolen submitted]). Therefore, the change in the selection dose may not have been a factor in the difference in ethanol metabolic rates in later generations. The most likely explanation is that the increased selection for sleep time and thereby the correlated (although unrelated, as we shall see) temperature drop was responsible for the apparent increasing differential in clearance rate. The other factor is, of course, that the initial studies with the mice were not nearly as thoroughly done with respect to dose, concentration, and so forth as the later studies have been. Had such studies been carried out earlier, they might have discovered these more subtle effects at that time. Smolen et al. (1986) have carried out a developmental study on alcohol and aldehyde dehydrogenates in SS and LS mice. Smolen et al. (1982) have studied aldehyde dehydrogenase distribution in these mice in more detail.

Other Metabolic Studies

French et al. (1979) found that 3-methylcholanthrene induced cytochrome P450 to a greater extent in SS than in LS mice. This finding included the microsomal ethanol-oxidizing system (MEOS) as well as aniline hydroxylase and aromatic hydrocarbon hydroxylase but not ethylmorphine *N*-demethylase or testosterone hydroxylase. Basal levels of cytochrome P450 were not different. Ethanol treatment also increased the cytochrome P450 level in SS mice to a greater extent than in LS mice. Hjelle et al. (1981) extended these findings and also studied kidney cytochrome P450 activity, with similar results. (See also Petersen and Atkinson 1979.)

Howerton et al. (1983b) measured the rate of disappearance of pentobarbital in these mice. They reported that [^{14}C]pentobarbital disappeared more rapidly from body fat of LS mice than from body fat of SS mice. The rates of disappearance of methylprylon were identical in the two lines. O'Connor et al. (1982) reported that pentobarbital disappeared more rapidly from the brain and blood of LS than of SS mice. The volumes of distribution were the same, however.

Correlated Behavioral Differences

Preference

Church et al. (1979) demonstrated that given a choice between glucose-saccharin and the same solution with 4 percent ethanol, the SS mice consumed more ethanol than did LS mice. Fuller (1980a) reported essentially the same findings (SS mice, 9.45 g/kg/day; LS mice, 7.14 g/kg/day). A study carried out by Erwin et al. (1980) with HS mice, on the other hand, failed to find any correlation between preference and brain sensitivity. The methods were quite different, however. Preference was measured in a free-choice situation, using 10 percent ethanol in water and a 15-day trial. Brain sensitivity was assessed by determining blood ethanol at the time animals could again balance themselves on a wooden dowel. Were it not for differences in technique, one would classify the original finding of Fuller as a false-positive, since it does not stand up to the test in other lines or strains of animals. Also, there is the lack of a correlation in inbred rats between sensitivity and preference (Spuhler and Deitrich 1984).

Dudek (1982) found that SS and LS mice do not differ in conditioned taste aversions.

Dependence

Using SS and LS mice of the 17th generation, Goldstein and Kakihana (1975) found that LS mice had a much milder withdrawal reaction than did SS mice after 3 days of exposure to ethanol vapors. They did not find any correlation in individual HS mice between sleep time and withdrawal severity, however. Again, this result would classify the finding in the selected lines as a false-positive.

Sanders (1980) demonstrated that 24 hr after an anesthetic dose of ethanol, both lines were most susceptible to fluorthyl-induced clonus, but only the LS mice were more susceptible to myoclonus. However, the SS mice were already as sensitive to myoclonus produced by fluorthyl as the LS mice became after ethanol treatment. This may have been a floor effect.

It is of interest that Horowitz and Allan (1982) found that SS mice exhibited more severe naloxone-precipitated morphine withdrawal than did the LS mice.

Tolerance

In designing experiments to determine whether the SS and LS mice differ in chronic or acute tolerance to ethanol, one is faced with a dilemma. One can give the same test dose of ethanol to both SS and LS mice, but then the pharmacologic effects are markedly different. Does one calculate the tolerance developed on a percentage increase in the baseline values or on the absolute decrease in CNS sensitivity? On the other hand, the administration of different doses is not at all satisfactory either.

Several investigators have approached this problem. Tabakoff et al. (1980) found that SS mice developed chronic tolerance more rapidly than LS mice after daily treatment with a total of 9.2 g/kg/day in two doses. No evidence was found for acute tolerance, using blood levels at the loss and regaining of the righting reflex, in this or a previous study (Tabakoff and Ritzmann 1979). The dilemma is well illustrated in the latter paper. The LS mice had an initial sleep time of 156 min. After 5 days of treatment, they had a sleep time of about 55 min, or 35 percent of the original value. This difference represented a loss of 80 min in CNS sensitivity. On the other hand, the SS mice had an initial sleep time of 15 min and were completely tolerant at the end of 5 days of testing, which represented a 100 percent loss in sensitivity, but only a 15-min loss in sleep time. Practically speaking, one cannot go much higher in the doses for LS mice and have them survive, but a sleep time of more than 15 min for SS mice would be desirable. In an attempt to circumvent these problems, C. J. P. Eriksson, R. C. Baker, and R. A. Deitrich (unpublished data) used daily doses of 2.5-5.5 g/kg/day for LS mice and 3.5-6.5 g/kg/day for SS mice. Sleep times, body temperature, and blood ethanol upon awakening were determined each day after the test dose.

If one plots the blood ethanol upon awakening as a function of the initial dose of ethanol for naive animals, the slope for SS mice is 6.27, with a nonsignificant (NS) correlation coefficient of 0.14. For the LS mice, however, the slope is 14.62 and the correlation coefficient is 0.47, which is significant at $P < 0.02$ ($n = 29$). This finding would indicate that there is some

development of acute tolerance, since with the larger doses the sleep time is proportionally longer. For animals that have been treated for 5 days, the slope for SS mice has increased to 24.9, with a correlation coefficient of 0.60; for LS mice, the slope is 20.4, with a correlation coefficient of 0.64. These results indicate that the SS mice have developed a large amount of tolerance (slope change from 6.27 to 24.9) after 5 days of treatment. The LS mice, on the other hand, have only been able to maintain or slightly improve the acute tolerance that they had on day 1. Thus, these results agree with those of Tabakoff et al. (1979, 1980) in that the SS animals developed the greater amount of chronic tolerance. They do not agree in that we observed acute tolerance in the LS animals, albeit by using a different technique. Smolen and Smolen (submitted) have pointed out that brain levels of ethanol are higher than blood levels up to 15 min after IP administration. Thus, the technique of taking blood alcohol only at the loss and again at the regain of the righting reflex is a flawed one. Parsons et al. (1982) found that SS mice developed rapid tolerance to the ataxic effects of ethanol, as tested by dowel balance technique. The LS mice developed this type of tolerance more slowly but achieved approximately the same degree of tolerance. In both cases, there was an increase in erythrocyte levels of chlorestrol when tolerance was produced.

Deitrich (unpublished results) found that the degree of tolerance, as measured by blood alcohol levels at the time that mice could remain on a wooden dowel, was significant only for LS mice and only when 1.5, not 2, g/kg of ethanol was used as the starting dose.

Palmer et al. (1985) found that tolerance to ethanol could be demonstrated at the level of the rate of firing of cerebellar Purkinje cells. Upon chronic treatment of mice with ethanol, the Purkinje cells were significantly tolerant to pressure-ejected ethanol; however, there was no difference between the SS and LS lines in these experiments.

Erwin et al. (1980) tested the relationship between sensitivity and acute tolerance measured by the dowel balance test in HS mice and found a significant (-0.34) negative correlation.

The consensus from these experiments seems to be that SS mice develop chronic tolerance to a greater degree than do LS mice. Whether there is a

significant development of acute tolerance is somewhat less certain and seems to depend on the technique used to determine this effect. Except for the acute tolerance studies by Erwin et al. (1980), these findings have not been pursued.

Activation by Ethanol

The activating effect of ethanol at low doses is of considerable interest because it may be related to the reinforcing properties of ethanol. Sanders (1975) was the first to study this effect in SS and LS mice. She found that SS mice were more active than LS mice in an open field after receiving 1.4, 1.8, or 2.0 g/kg of ethanol. That this effect was not due simply to the greater depressant effect of ethanol in the LS mice was indicated by the same performance of the lines on a rotarod at these doses. The SS mice were more sensitive to pentobarbital in the open-field test but only at doses of 16 mg/kg and above. Subsequently, Sanders et al. (1978) found that SS mice were more sensitive to activation by low-dose paraldehyde than LS mice but were less sensitive to this compound when tested by sleep time. Conversely, the SS mice were also less sensitive to trichloroethanol in the sleep time test than LS mice but did not differ in the activating effect produced by this compound. When this effect was tested in the HS population, "there was no relation between the degree of activation produced by low dose of ethanol and sensitivity to the hypnotic effects of a higher dose." On the other hand, Dudek and Abbott (1984b) used an F₂ cross between SS and LS B-A mice and found a significant negative correlation (-0.36) between sleep time and activation. It should be remembered that these mice have not undergone any selection pressure since generation 18.

Dudek et al. (1984b) also found that SS mice were more sensitive to the activating effects of ethanol. *t*-Butanol elicited similar reactions. F₁ hybrids showed intermediate inheritance for the trait.

Grooming behavior is also increased in SS mice at low-dose ethanol levels (Allan and Isaacson 1985). This effect may be related to increased levels of adrenocorticotropin (ACTH).

The negative correlation between sleep time and activating effect originally observed in SS and LS mice and also in the F_2 cross would be classified as a positive finding. The failure to confirm this result in the HS mice leaves some doubt as to the true situation. In any case, this matter does not appear to have been pursued further but may be a fruitful area for further research.

Anticonvulsant Effect

Sanders and Sharpless (1978) demonstrated that SS mice were more susceptible to myoclonus produced by flurothyl but that although ethanol antagonized the convulsive effect of flurothyl, it did it equally well in both lines. This effect was significant at low doses (0.35 g/kg) of ethanol. Since the SS mice were much more susceptible to flurothyl to begin with, it was necessary to administer equimyoclonic doses of flurothyl to the animals. If this is not done, it appears that the SS mice are better protected from myoclonus than are the LS mice.

Using procedures essentially identical to those used by Sanders and Sharpless, Greer and Alpern (1978) confirmed that SS mice were more susceptible to flurothyl-induced myoclonus, but in contrast to the findings of Sanders and Sharpless, they found that the SS mice were much less susceptible to clonus than were the LS mice. They went on to study the influence of a number of neurotransmitter agonists and antagonists in these lines of mice with relationship to the latency to myoclonus or clonus precipitated by flurothyl. They found that dopaminergic drugs (apomorphine and haloperidol) affected only myoclonus, whereas cholinergic agents (pilocarpine and scopolamine) and GABAergic drugs (aminooxyacetic acid para-chlorophenylalanine [AOAA] and bicuculline) and the serotonin depletor PCPA all affected clonus. Noradrenergic drugs altered both types of convulsions. Identical data for apomorphine, haloperidol, pilocarpine, and bicuculline had been reported in a previous publication (Greer and Alpern 1977).

A paradoxical effect of amphetamine was observed in SS mice (Greer and Alpern 1980). In these mice but not in LS mice, amphetamine induced a *decreased* seizure susceptibility between 15 and 35 days of age. Amphetamine in LS mice always caused an *increased* seizure susceptibility.

It was suggested that SS mice might serve as an animal model of preadolescent hyperkinesis. There is evidence that this effect is mediated by the dopamine system (Alpern and Greer 1977). (See also Smolen and Smolen 1986.)

It seems clear that the anticonvulsant effect of ethanol is not related to the ataxic effect in these lines. This is a negative finding.

Hypothermia

Moore and Kakihana (1978) were the first to document the differential response of SS and LS mice to the hypothermic effect of ethanol. They gave a dose of 2 g/kg to these lines and observed a greater drop in temperature and a slower return to normal in the LS mice. At a dose of 1 g/kg, however, there was a significant hyperthermia in B-A SS mice as compared with LS mice. This finding might have been related to the greater activating effect of ethanol in the SS mice. Brick and Horowitz (1982) did not observe a significant hyperthermia with low-dose ethanol.

Baker et al. (1980) gave a larger dose of ethanol (4.1 g/kg) and observed a larger temperature drop in the LS mice than in the SS mice. Interestingly, although halothane caused hypothermia, it did not do so differentially in the SS and LS mice.

Brick and Horowitz (1982) using the Binghamton-Albany (B-A) lines of mice also found a differential hypothermia between the lines. They made the interesting observation that SS mice were more sensitive to the hypothermic effects of morphine but only 30 min after morphine administration. Naloxone blocked morphine hypothermia in both lines but blocked ethanol-induced hypothermia only in SS mice. Also, SS mice exhibited cross-tolerance to ethanol-induced hypothermia after chronic treatment with morphine (Brick and Horowitz 1983).

Howerton et al. (1983b) demonstrated that propanol, butanol and 3-methyl butanol all produced hypothermia in SS and LS mice but that the response was different between the two lines only for ethanol, whereas the sleep time was differentially affected by all alcohols in the SS and LS lines. This finding indicates that the hypothermic response is under different genetic control

than is the sleep time effect. Similar conclusions were reached by C. J. P. Eriksson (personal communication) when he attempted to correlate sleep time with temperature drop in a group of HS mice and found no correlation. Likewise, A. Collins (personal communication) has found no correlation between the two in a series of inbred strains. The recombinant (LS and SS) inbred strains should also be useful in resolving this question.

The question of what effect hypothermia has on sleep time or other responses is an important one. Romm and Collins (1987) found that hypothermia was responsible for decreased ethanol metabolism in LS as compared with SS mice. Erwin et al. (1987) have maintained animals at normal temperature and measured sleep time after ethanol administration; they find that the sleep time differential still persists.

A large number of compounds cause differential hypothermic response in the SS and LS mice. French et al. (1985a) observed that α -methylparatyrosine (AMPT) had this property. T. Pang, R. A. Deitrich, and C. Melchior (unpublished results) investigated a number of compounds for their effects on body temperature of SS and LS mice. Apomorphine and oxotremorine caused a differential hypothermic response in the two lines, the LS mice being more sensitive. Clonidine caused equal hypothermic responses in both lines; pentobarbital caused a hypothermic response, but in this case the SS mice were more sensitive. Deitrich et al. (in press) found that phorbol esters caused hypothermic response differentially in the two lines. On the other hand, neurotensin (Erwin and Su 1989) caused an equal depression of body temperature in the two lines. A detailed study of the relationships between the hypothermic and anesthetic effects of ethanol and various other agents should yield promising results concerning both the mechanism of action of ethanol and mechanisms controlling body temperature.

Differences in the Absence of Ethanol and Prenatal Effects

There are some easily observed differences between LS and SS mice before ethanol is given. The LS mice are much more active in the cage and give rise to the axiom "Long-sleep mice will give you dirty knees" (A. Smolen) because they escape readily when the cage top is opened and are exceedingly fast (and

hence difficult to catch). On the other hand, few controlled studies have been carried out with the animals. The most often asked question is, "What is their sleep pattern before ethanol is given?" No published data are available, and apparently no one has studied this matter.

SS mice are more fertile than LS mice and have a higher sexual activity (Baer and Crumpacker 1977). The effect of administration of ethanol during gestation and lactation as a 10 percent solution in the drinking water caused decreased survival of LS but not SS pups. Increased cannibalism by LS mothers contributed to this effect. In an experiment in which the dams received ethanol only during days 2-14 of lactation, Swanberg and Wilson (1979) also found that the LS dams were poorer mothers and had fewer surviving pups despite their lower consumption of the 15 percent ethanol in the drinking water.

Using the B-A colony of SS and LS mice, Gilliam et al. (1987) found that prenatal treatment of SS and LS mice led to the LS mouse pups requiring more trials to reach criterion in a passive avoidance test but not in a number of other tasks. They concluded that any fetal alcohol effects may be task specific in these and probably other animals.

Effect of Other CNS Depressants

Pentobarbital is discussed separately because studies with it have raised considerable disagreement among workers in the field. Originally, Erwin et al. (1976), using generation 16 mice, reported that there was no significant difference between the sleep time produced by a pentobarbital dose of 60 mg/kg. The ratio of LS/SS sleep times was 0.67. Reanalysis of these data by Alpern and McIntyre (1985a) showed that there was in fact a significant difference between LS and SS mice at $P < 0.01$. A study by Siemens and Chan (1976) using IBG mice of generation 18 found that SS mice had a longer sleep time than did LS mice (LS/SS sleep time=0.50) but that the brain levels at awakening were the same after a dose of 50 mg/kg. Furthermore, they determined that the apparent volume of distribution of the drug was greater in LS than in SS mice. The half-lives for pentobarbital were the same in the two lines. Sanders et al. (1978) gave 60 mg/kg of sodium pentobarbital (54 mg/kg of free acid) to LS and SS mice of generations 19-21 and failed to

find a difference in sleep time (LS/SS sleep time=0.79, $n=10$, $P=NS$). Ryan et al. (1979) studied the effect of pentobarbital on the electroencephalogram (EEG) and could detect no differences between lines when the animals were given 50 mg/kg of pentobarbital as sodium pentobarbital (presumably). In 1982, O'Connor et al. again found that the SS mice were more sensitive to pentobarbital at doses ranging from 35 to 70 mg/kg. At a dose of 60 mg/kg, the LS/SS sleep time ratio was 0.51. Now, however, they found a significant difference in the rate of disappearance of pentobarbital from the blood of these lines. The LS mice had a much more rapid rate of metabolism than did the SS mice, but the volumes of distribution were the same, in contrast to the results of Siemens and Chan (1976). The net result is that there is no difference in brain sensitivity of the two lines to pentobarbital. These mice were of generations 28-31, or over 10 generations later than those used by either Erwin et al. or Siemens and Chan. Whether this fact explains the differences will probably never be known, since reconstruction of these early generations would be time consuming. Again in 1984, Dudek et al. (1984), using the B-A mice, found that pentobarbital (65 mg/kg) was more potent in SS than in LS mice (LS/SS ratio=0.61, $P<0.01$). Howerton et al. (1983b, 1984) also found that SS mice were more sensitive than LS mice at a range of doses for sleep time or temperature effects. Dudek and Phillips (1983) reported that SS mice were more stimulated by ethanol, methanol, t-butanol, and pentobarbital (30 mg/kg) than were the LS mice. In addition, they found that LS mice were more sensitive to the ataxic effect of pentobarbital at 20, 25, and 30 mg/kg. They made the point that "the previous assumption of specificity of the selection for alcohols may be a question of degree rather than a qualitative effect." This was the first report that there might be a differential effect of pentobarbital at low, nonanesthetic doses in the same direction as the effect of ethanol. Previously, Erwin et al. (1976) and Baker et al. (1980) had pointed out that the gaseous anesthetics did not differentiate between the lines of mice, indicating that the selection process had differentiated at least at that level of action and casting doubt on the long-held assertion that ethanol and the gaseous anesthetics acted by the same mechanism of action.

Papers by Alpern and McIntyre (1985a, 1986) and McIntyre and Alpern (1985, 1986a,b) attacked the idea that selection had been for alcohol differential sensitivity alone. These papers deserve detailed examination, since they go to the heart of the selection process. Alpern and McIntyre

(1985a) presented a dose response curve for sleep time, using doses of 25-55 mg/kg. They used five animals at each point but did not specify which generation of IBG mice was used. At each point, the LS animals slept longer than the SS animals except at 25 mg/kg, when no animals lost the righting reflex. Since the data were not homogeneous, a transformation was made and then a two-way analysis of variance was carried out which showed that the LS mice were more sensitive to pentobarbital. The most obvious problem with these data is that the authors ignored the already well-established difference in metabolism between these two lines of mice for pentobarbital. The other problem is that they used only five animals per point. A single bad injection in any group of five animals could have introduced a 20 percent error in the data that could not have been detected because no blood levels of pentobarbital were taken. The other problem is that the doses were below or only slightly above the ED_{50} values for pentobarbital in these animals (Erwin et al. 1976). Thus, at all but the highest dose, and perhaps not even then, not all animals lost the righting reflex. This means that a zero sleep time must have been entered for such animals. However, the brain sensitivity of such animals is unknown. Brain sensitivity is not determinable if the mice did not sleep nor if the blood level of pentobarbital was not measured. One has no way of knowing what dose of pentobarbital would be required to cause such an animal to lose the righting response. This problem is exacerbated by use of only five animals per point. The study by Erwin et al. (1976), though using only a single dose, used 70 SS and 56 LS mice. This problem of how to handle nonsleeping mice is not unique to this study, but it apparently was unrecognized by the authors.

In any case, the problem appears to have been put to rest by a large study carried out by Marley et al. (1986) in which the " ED_{60} " values for a large series of compounds in SS and LS mice were determined. ED_{60} is defined as the dose of a compound necessary to achieve a 60-min sleep time in the SS and LS mice. By plotting the ratio of ED_{60} for LS mice/ ED_{60} for SS mice as a function of lipid solubility, they obtained a linear relationship with a negative slope, which indicated that the selection process for LS and SS mice favored those CNS depressants that were more water soluble. Lipid-soluble compounds either showed no difference in ED_{60} ratios or had a value of less than 1, indicating the SS animals are more sensitive than LS animals to these compounds. These results correlate nicely with studies discussed below,

which indicate that the selection process has favored drugs that act at the cell surface rather than more deeply in the membrane. (See also earlier studies by Howerton et al. [1982a,b, 1983a,b, 1984] and Khanna et al. [1984].)

Another definitive study was carried out by Allan and Harris (submitted), who determined the correlation between pentobarbital and ethanol sleep time in a large group of HS mice and found no correlation between the two.

Studies on the Mechanism of Action of Ethanol

Electrophysiology

Although there had been one study carried out with EEG in SS and LS mice (Ryan et al. 1979), it was not until 1980 that Sorensen et al. (1980, 1981) used single-unit recording in these mice. They found that the Purkinje cells of urethane-anesthetized SS and LS mice fired spontaneously at about the same rate but that the sensitivity of these cells in LS mice to locally applied ethanol was some 30-fold greater than that of SS cerebellar Purkinje cells. While ethanol also depressed the firing rate in the hippocampus pyramidal cells, it did so to the same extent in both lines of mice. This development has been thoroughly investigated since that time. In any finding of this type where there is a difference between the two lines, there is the danger that the result is a false-positive. Spuhler et al. (1982) recorded sleep time and cerebellar Purkinje cell sensitivity in individual mice from the eight inbred strains that made up the original HS line. They found a reliability coefficient of 0.96, a correlation of -0.997, a heritability for sleep time of 0.53, and a heritability for Purkinje neuron firing inhibition of 0.76. Similar results have also recently been obtained with a rat selection for sleep time (Johnson et al. 1985; Palmer et al. 1987a).

This observation has been vigorously pursued. It has been found that mouse fetal cerebellar tissue transplanted to the anterior chamber of the eye of a mouse and allowed to develop retains the sensitivity of the donor line regardless of whether it was transplanted to SS or LS mice (Palmer et al. 1982; Seiger et al. 1983). The LS cells were approximately 10-fold more sensitive than were the neurons from SS mice. Basile et al. (1983) demonstrated that this sensitivity was maintained in vitro in cerebellar slices

and that it appeared to be relatively independent of synaptic transmission, since it was observed in the presence of low calcium, high magnesium levels that would suppress synaptic events. In this case, the cells from LS mice were about fivefold more sensitive than those from SS mice. It is interesting that in the whole animals the LS mice were about twofold more sensitive than the SS mice, indicating that the sensitivity of the cerebellar Purkinje cells was greatly modulated by other neuronal mechanisms in producing the observed behavior of prolonged sleep time in the LS mice.

Palmer et al. (1984) also carried out cerebellarectomies in neonatal mice of the SS and LS lines. They found that the SS animals were more LS-like as adults but that there was no effect in LS animals. This finding would indicate that the cerebellum is important in the response of SS mice but that other CNS mechanisms control the sleep time in LS mice, perhaps because the cerebellum is so sensitive that it does not recover significant function in the LS mice until long after other mechanisms have caused these mice to right themselves. It is not uncommon to find LS mice still on their backs at a time when they are perfectly capable of righting themselves and escaping if they are startled. One obvious problem with such studies is that the pups grow to adulthood missing all or part of their cerebellum, and other brain structures assume many of the functions normally carried out by the cerebellum. Nevertheless, this finding again illustrates the marked effect that the selection process has had on these mice.

These studies have directed many subsequent investigations toward the cerebellum, and particularly to the Purkinje cells. It is important to emphasize that although these findings are among the most conclusive, not all of the variance between SS and LS mice can be accounted for. Whatever the difference between Purkinje cells in the two lines, other differences between SS and LS mice have also been found, but the interrelationships between these findings are unknown.

Membranes

Given the popularity of the membrane hypothesis of the action of ethanol, it was hypothesized that the function and thus composition of neuronal cell membranes should be different in the two lines of mice. Only two reports have been published despite a large number of negative, and thus

unpublished, studies. Koblin and Deady (1980, 1981) studied the effect of gaseous anesthetics in these mice and found that SS mice required 34 percent more nitrous oxide and 20 percent more enflurane than did LS mice in a rolling test in an anesthetic chamber. These results with very lipid soluble substances would not agree with the finding of Marley et al. (1986) that lipid-soluble agents do not discriminate or discriminate in the reverse direction between these lines. They did not, however, find any differences in lipid composition between the lipid content of synaptic membranes isolated from whole brain of the two lines except for the level of arachadonate, which was higher in LS mice. Baker (1987) reinvestigated this question, but in addition to performing whole-brain assays, he also studied synaptic membranes from cerebellum. Again no differences were found, nor were any differences detected after chronic treatment of these mice with ethanol.

Ullman et al. (1987) found that GM₁ ganglioside was higher in the cerebellum and in cerebellar synaptic plasma membranes of LS mice as compared with SS mice. However, there was no difference in GM₁ concentration in whole hippocampus between the two lines. There was no difference in the molecular species of GM₁ between the lines in any case.

In contrast to these negative findings on lipid composition, a number of studies on cell membrane function and physical properties have been carried out. Goldstein and colleagues (1982) used electron spin resonance to study the cell membrane fluidity of membranes from SS and LS mice. They found no baseline difference between the lines; however, the membranes from LS brains were more sensitive to the fluidizing effect of ethanol. This effect was duplicated when the extremes of sleep times from an HS population were studied. That is, the SS-like and LS-like mice reacted identically to the mice selectively bred for many generations. This finding suggests two possibilities: (1) only a few genes control this trait, and the fact that it took many generations to select for SS and LS mice is evidence that cell membrane fluidity has nothing to do with initial CNS depressant effect of ethanol; and (2) this trait was selected very early in the selection process but a great many other, less important factors modify the righting reflex, and the subsequent generations of selection were necessary to select for these modifying factors.

Two other factors are important in these experiments, as discussed elsewhere in this volume. The cell membrane-fluidizing hypothesis has a number of

pros and cons, chief among which is the fact that a small rise in temperature achieves the same fluidizing effect as a lethal dose of ethanol. The second factor is that subsequent experiments with electron spin or fluorescent probes, which sampled the cell membrane in the center rather than at the surface, failed to detect any difference between SS and LS brain membranes (Harris et al. 1987, 1988; Pearlman and Goldstein 1984). This observation agrees well with the finding of Ullman et al. (1987) that GM₁ ganglioside, located on the cell surface, is quantitatively different in the SS and LS mouse brains.

The problem of cell membrane fluidity in microenvironments cannot be approached by these techniques. However, one may obtain some information by studying the activity of enzymes that are embedded in the cell membrane. This assay has been carried out for several such enzymes. Marks et al. (1984) studied the high- and low-ouabain-sensitive brain ATPases in SS and LS mice and found that ethanol inhibited the enzymes from the two lines to the same extent. In a more detailed study of these as well as other enzymes, Collins et al. (1984) found that the transition temperature for the high-ouabain-sensitive ATPase was reduced twice as much by ethanol in the membranes from LS mice as in those from SS mice. The transition temperature in the absence of ethanol for the low-ouabain-sensitive enzyme was higher in SS membranes. This was also true for the transition temperature of acetylcholinesterase, which was higher in SS membranes by 2.3° C. These are relatively large differences in transition temperature and must be related to differences in the structure of either the enzyme or the lipid matrix in which it exists.

Neurotransmitters

GABA

Some of the most interesting results have been obtained from a study of the GABA system. Chan (1976) found that SS and LS mice had equivalent amounts of GABA in the brain and that the increases in GABA seen after ethanol administration were similar in the two lines. Howerton and Collins (1984a) found that potassium-stimulated release of GABA in brain slices was inhibited by ethanol to a greater extent in LS than in SS mice but that

inhibition of GABA uptake by ethanol was not different in the two lines (Howerton et al. 1982a), nor was ethanol inhibition of GABA release (Howerton and Collins 1984a). Investigations then turned more to the function of the GABA receptors and away from GABA itself. Martz et al. (1983) observed that the agonists tetrahydroisoxazol-pyrideneol and baclofen had a greater effect by themselves in LS than in SS mice; i.e., the ED_{50} values were greater in the SS mice for the incoordinating effect of these compounds. At about the same time, Masserano and Weiner (1982) determined that picrotoxin administered intracerebroventricularly (ICV) would antagonize ethanol-induced anesthesia but equally well in both SS and LS mice.

Allan and Harris (1986, 1987) made the seminal observation that the GABA agonist mucimol would stimulate chloride uptake in synaptoneurosomes to a greater extent in LS than in SS mice and that ethanol enhancement of this effect was greater in LS than in SS mice. Similar results have been obtained with selected rat lines (Allan et al. submitted). (See also Allan et al. [1987 and 1988a] for a study on mice selected for benzodiazepine sensitivity.) The number and K_d values of benzodiazepine receptors are the same in the two lines (Marley and Wehner 1987), however.

GABA enhancement of flunitrazepan binding is greater in LS than in SS mice (McIntyre et al. 1988). Also, GABA displacement of *t*-butylbicyclophosphorothionate (TBPS) binding is greater in LS than in SS mice. That there is some structural difference in the receptors between SS and LS mice is illustrated by the finding that the heat inactivation curves show that LS receptors are more labile than SS receptors (Marley and Wehner 1988; McIntyre et al. 1988). GABA protects against heat inactivation better in LS than in SS mice (Marley et al. 1988). Miller et al. (1988), using in vivo binding techniques, found that binding of Ro 15-1788 was greater in the cortex and hippocampus of LS than of SS mice but that clonazepam binding in the presence of ethanol was greater in SS than in LS mice.

Several lines of evidence indicate that the GABA-benzodiazepine chloride channel complex may be more closely related to seizure susceptibility than to sleep time. One indication is that latency to seizure with 3-mercaptopropionic acid is greater in SS than in LS mice (Marley and Wehner 1987). Marley and Wehner (1987) found a correlation between enhancement

of flunitrazepam binding by GABA and seizure susceptibility. Stinchcomb et al. (in press) studied the effect of Ro 15-4513, a partial inverse agonist for benzodiazepine receptor in these mice. (See also the discussion above concerning seizure susceptibility.) A more thorough discussion of this topic can be found in the papers by Wehner et al., Harris and Allan, Olsen et al., and McIntyre et al. (this volume) as well in those by Marley et al. (1988, in press).

CATECHOLAMINES

Several investigations with SS and LS mice have been undertaken to elucidate the possible role of catecholamine neurotransmitters in the effect of ethanol. Dibner et al. (1980) studied the level and affinity of β -adrenergic receptors as well as both basal and activity-stimulated adenylate cyclase in various brain areas. (See also Rabin and Molinoff 1979.) Although some differences were found, they did not correlate with the behavioral differences between SS, LS, and HS mice. These differences at this point are false-positives, since the values for HS mice do not fall between those of SS and LS, as do the sleep time values. The danger of using only HS mice as a control is that unless many HS mice from different litters are used, a large number of SS-like or LS-like mice may be obtained.

Two studies have been carried out with amine-depleting agents. Erwin and Cornell (1986), using 6-OH dopamine (6-OHDA) in SS and LS mice, observed that whereas ethanol-induced sleep time increased in treated SS mice, there was not a corresponding drop in blood ethanol at awakening. They achieved depletions ranging from 49 to 81 percent in various brain areas. Similar results were obtained by Spuhler et al. (1987), using N-(2-chloroethyl)-N-ethyl-2 bromobenzylamine (DSP4) in SS and LS mice. They achieved depletions of norepinephrine of 30-85 percent depending on the brain area, without alteration of dopamine or serotonin levels. They found no alteration of ethanol-induced sleep time in either SS or LS mice.

Erwin and Cornell (1986) observed that pretreatment by 6-OHDA attenuated the ethanol-induced fall in body temperature in both lines as well as the ethanol-induced rise in blood glucose. Since both of these effects are greater to begin with in LS mice, the attenuation by 6-OHDA pretreatment appears to be more dramatic in LS than in SS mice.

In all experiments such as that by Erwin and Cornell (1986), the question of how much depletion is required to achieve a behavioral effect cannot be answered if the results are negative. Another problem is that often differential depletions are found in different mouse lines.

Masserano and Weiner (1982) tested the effects on sleep time a number of adrenergic agonists and antagonists given ICV. Both norepinephrine and dopamine reduced sleep time in LS mice, but in SS mice sleep times were increased by norepinephrine, dopamine, epinephrine, and isoproterenol. Clonidine, an α_2 agonist, increased sleep time in both lines, whereas phentolamine, an α agonist, increased sleep time in LS but not SS mice. SS mice proved to be much more sensitive to reserpine, since 92 percent of them died during 10 days of treatment with a 5-mg/kg dose of reserpine and only 13 percent of LS mice died. The surviving LS mice had a significant increase in tyrosine hydroxylase (TH) in the cerebellum and locus ceruleus. Allan et al. (1984) found that propranolol would decrease sleep times of LS but not SS mice. Phentolamine had no effect. They used the B-A group of mice and gave the drugs systemically.

The problem encountered in experiments such as these is that the half-lives of the various compounds are extremely varied. Sometimes the half-lives may be only a small fraction of the sleep times achieved. Many of these findings should be followed up by using blood ethanol at loss of the righting reflex after intragastric administration to more closely approach the half-lives of some of the more labile compounds.

A number of studies on the effects of TH in these mice have been undertaken by N. Weiner and his group. An early observation was that TH increased in the hypothalamus after ethanol administration at the time of regaining the righting reflex in both SS and LS mice. Of course, this is a much higher blood alcohol level and at a much shorter time than for SS mice (Baizer et al. 1981). French et al. (1985a) used AMPT, an inhibitor of TH, to deplete brain catecholamines in SS and LS mice. LS mice were more sensitive to the depleting effects as well as to the hypothermic effects of AMPT. This latter finding was a result of different pharmacokinetics of AMPT in the two lines, since brain and plasma levels of AMPT were higher in LS mice. French et al. also found that TH from LS mice had a lower

affinity for tyrosine. There is also a difference in tyrosine availability in SS and LS mice (French et al. 1988a).

French and Weiner (1984) also determined the effect of ethanol on TH *in vivo* by measuring the levels of DOPA after administration of a DOPA decarboxylase inhibitor. They found no differences in basal catecholamine levels or TH in any brain region except in cerebellum, where the norepinephrine levels and TH rates in SS mice were higher. Ethanol decreased TH rates in the cerebellum, but the TH rate in SS mice returned to the control value more quickly than in LS mice. In contrast, they found that TH, epinephrine, and norepinephrine levels in the adrenal glands were higher in LS than in SS mice (French et al. 1985b). Ethanol administration increased TH levels in SS adrenal glands but decreased them in LS adrenal glands; in the latter, TH persisted even after levels in the SS mice had declined to normal. The adrenal TH increase in SS mice was accompanied by an increased affinity for the TH pterin cofactor. The decreased TH levels in LS mice were associated with decreased affinity of the cofactor. Blockade of neuronal input with chlorisondamine had no effect on the adrenal TH levels or sleep time in LS mice but blocked the increase in TH levels in SS adrenal glands and prolonged SS sleep time. Zgombick et al. (1986) observed that a dose of ethanol produced a much increased plasma catecholamine level in LS mice but a much smaller increase in SS mice. These findings and the relationship to blood glucose increases are discussed below in the section on hypothalamic-pituitary-adrenal effects. See French et al. (1988a) for a more detailed study.

The many interesting observations made in this area are classified as probable since they have not yet been verified in other lines or strains, although recent work with the selected rat lines provides support for many of these observations (see Spuhler et al., this volume).

CENTRAL SYMPATHETIC OUTFLOW

It was recognized very early by Kakihana (1976b) that ethanol had a differential effect on adrenal cortical steroids in SS and LS mice. The relationship of this response to the concentration of ethanol used for selection (30 percent) is of some interest in that perhaps smaller

concentrations would not elicit such a marked increased in the adrenal cortex response.

Erwin and Towell (1983) observed that ethanol elicited a much greater hyperglycemic response in LS than in SS mice, that it was not produced by pentobarbital or halothane, and that it could be blocked by fasting, adrenalectomy, and administration of α - and β -adrenergic antagonists. Results obtained after ICV injections of ethanol indicated that the response was centrally mediated. Zgombick et al. (1986) then correlated these findings with plasma norepinephrine and epinephrine concentrations in the two lines of mice. They found excellent correlations between blood ethanol, plasma epinephrine, and plasma glucose in LS mice. SS mice were much less responsive in both plasma catecholamine and blood glucose levels. This response was blocked by the ganglionic blocker chlorisondamine. Again, neither pentobarbital nor halothane anesthesia produced the differential response in the two lines of mice. Thus, all available evidence points toward a central effect of ethanol in elevating plasma catecholamines and blood glucose. This effect appears to be due to an effect on central outflow of the sympathetic nervous system. No experiments have been carried out in other lines or strains of mice.

CHOLINERGIC-MUSCARINIC RECEPTORS

Masserano and Weiner (1982) found no effect of acetylcholine or atropine on sleep time. More recently, however, Erwin (1987) and Erwin et al. (1988) found that both carbachol and oxotremorine increased the sensitivity of SS but not LS mice to ethanol. Neither pirenzepine nor atropine, each a muscarinic antagonist, had any effect by itself on ethanol sleep time. However, both partially antagonized the oxotremorine-enhanced effect of ethanol on SS mice.

NICOTINIC RECEPTORS

The nicotinic antagonists tubocurare and hexamethonium decreased sleep time in LS but not SS mice. The effect in LS mice was blocked by atropine (Masserano and Weiner 1982). It was postulated that the block of the nicotinic receptors caused a reduced negative feedback on cholinergic neurons, increased cholinergic neuronal activity, and enhanced acetylcholine

release. The released acetylcholine would then be available to interact with muscarinic receptors and reduce sleep time in this way. Blockade by atropine would be expected. This interpretation is not consistent with the results of Erwin discussed above, where direct-acting muscarinic agonists increased sleep time in SS but not LS mice.

DeFiebre et al. (1987) found that LS mice were more sensitive to the convulsant effects of nicotine and that there were differences in α -bungarotoxin binding in the cerebellum and striatum but not the hippocampus of LS as compared with SS mice.

ADENOSINE

Proctor and Dunwiddie (1984) and Proctor et al. (1985) observed that an adenosine antagonist, L-phenylisopropyladenosine (PIA), had a greater sedative and hypothermic effect on LS than on SS mice. This result was correlated with a greater number of A1 adenosine receptors in the brain stem (Fredholm et al. 1985). Long-term treatment of LS and SS mice with nicotine infusions produces decreased sensitivity to nicotine-induced seizures to a greater degree in LS than in SS mice (DeFiebre and Collins 1988). Cyclic AMP production stimulated by adenosine interaction with A2 receptors was not different in the two lines. Proctor et al. (1985) extended these studies and found that LS mice were also more sensitive to an adenosine antagonist, theophylline. These differences were not attributable to differential metabolism or distribution of PIA and theophylline. LS and SS mice made tolerant to ethanol did not show cross-tolerance to PIA, suggesting "that genetic selection for ethanol sensitivity has resulted in parallel CNS sensitivity to purinergic drugs, but that acute alterations in sensitivity due to the development of ethanol tolerance do not involve changes in purinergic systems." These results have not been followed up in other lines or strains of mice.

GLUTAMATE

Disbrow and Ruth (1984) observed that the potassium-induced release of glutamate from slices of cortex and hippocampus from SS mice was greater than in LS mice. In hippocampus the difference was reversed, and no differences were seen in glutamate release from cerebellum. The release was

calcium dependent. They then studied the accumulation of labeled glutamate in synaptic vesicles from whole brains of these mice (Disbrow and Ruth 1985). Again, the vesicles from SS mouse brain accumulated glutamate to a greater degree than did vesicles from LS mice. Also, the endogenous glutamate in crude and enriched vesicles from SS mice was 1.5-fold higher than in vesicles from LS mice. Given that glutamate is an excitatory amino acid, these studies are of interest.

NEUROTENSIN

Neurotensin is covered in detail by Erwin (this volume). The pertinent references are Erwin and Jones (in press), Erwin (1986), Erwin and Korte (1988), and Erwin and Su (1988).

Hypothalamic-Pituitary-Adrenal Cortex

ADRENAL CORTEX

Kakihana (1976a,b) originally observed that LS mice responded more strongly than SS mice to a dose of ethanol, with a marked increase in plasma adrenal steroids. Zgombick and Erwin (1987) confirmed these findings and studied the phenomenon in more detail. They found that ICV-administered saline caused a stress-induced adrenocortical response that was equal in both lines of mice. A number of agents given ICV modified the ethanol-induced adrenocortical response. Clonidine, an α_2 -adrenergic agonist, blocked ethanol-induced elevations in plasma corticosterone at doses ranging from 1 to 10 μ g per mouse. Only the highest dose blocked the response in SS mice. Yohimbine, an α_2 antagonist, induced a marked elevation in plasma corticosterone only in LS mice and reversed the effect of clonidine on the ethanol-induced rise in corticosterone. Carbachol caused increased corticosterone levels in both lines, but atropine and hexamethonium did not alter the ethanol-induced response. Zgombick and Erwin (1987) found that the response was centrally mediated and blocked by administration of antibodies to corticotropin-releasing factor (CRF). Both LS and SS mice had similar increased plasma corticosterone levels after CRF or ACTH administration. This response was obviously correlated with the sleep time; however, confirmation of the causal relationship awaits further studies,

probably in the RI strains now available. (See also Swanberg 1977 and Swanberg et al. 1979.)

THYROID

Disbrow et al. (1986) reported that the thyroid status as measured by the free thyroxine index was significantly higher in SS than in LS mice at day 10 through day 65. The difference was maximal between days 11 and 14 postpartum. It is also at this time that there are rapid changes in the CNS sensitivity to ethanol. Pups up to 21 days of age are remarkably sensitive to the effects of ethanol, and the differences between SS and LS mice are evident even at this time. (W. Keir and R. A. Deitrich, unpublished results.) Disbrow et al. found that postpartum pups were very sensitive to ethanol but that the sensitivities in SS and LS mice were the same at day 9 and began to diverge after that time. No explanation for this discrepancy in the two sets of results has been found to date. (See also Weiner et al. 1987.)

Calcium

The effects of calcium on the CNS are numerous. It has been observed that calcium given ICV to SS and LS mice has a differential effect on the ethanol-induced sleep time. ICV-administered calcium chloride or a calcium ionophore increased the sensitivity of SS mice to ethanol, as measured by the blood alcohol level at the time of loss of the righting response (Morrow and Erwin 1986). Neurotensin had a similar effect when given ICV, and concurrent administration of calcium and neurotensin also increased sensitivity of SS mice in an additive manner. β -Endorphin increased ethanol sensitivity in SS mice, but concurrent administration with calcium produced no additional sensitivity (Morrow and Erwin 1987). Palmer et al. (1987b) carried this work a step further when they measured the ethanol sensitivity of cerebellar Purkinje cell firing rates under conditions whereby calcium was given ICV or directly applied to the cell by pressure ejection. They found that the sensitivity of Purkinje cells in SS but not LS cerebella to locally applied ethanol was greatly increased by calcium. This finding is a remarkable demonstration of the correlation between behavioral observations and cellular mechanisms. Again, these observations should be further characterized in other lines, strains, or species of animals to confirm the cause-effect relationship.

Daniell and Harris (in press) found that inositol trisphosphate (IP3) and ethanol released calcium from separate stores of brain microsomes. In cerebral cortex microsomes, ethanol released calcium to a greater degree in microsomes from LS mice than in those from SS mice. Chronic ethanol treatment enhanced IP3-mediated calcium release from brain microsomes but did not change ethanol-stimulated calcium release from either brain or liver microsomes. They concluded that ethanol's depressant action might be related to calcium release but that tolerance and dependence apparently were not. These results should be compared with those of Morrow and Erwin (1986, 1987), who found that calcium given ICV caused an increase in sleep time in SS but not LS mice. These two approaches obviously differ with respect to where the increased calcium is, and much more work needs to be done to delineate the role of calcium in the actions of ethanol.

Prostaglandins

A. C. Collins and his group have carried out extensive studies on the relationship between the effect of prostaglandin synthesis inhibitors and the actions of ethanol. The initial observation was made with inbred mice, in which it was observed that indomethacin inhibited the depressant effect of ethanol (Collins et al. 1985; George et al. 1982, 1986). They extended these studies to the selectively bred mouse lines. They first found that indomethacin pretreatment reduced the mortality rate and also the number of animals that lost the righting reflex at a dose of ethanol that was lethal to 69 percent of mice in the SS line. George et al. (1983) extended these studies to include LS mice as well. They found that LS mice required higher doses of indomethacin to antagonize the depressant effect of ethanol. Once that factor had been taken into account, the compound reduced sleep time equally well in both lines. There were several aspects of the time of pretreatment that were also important, however. In a large study, George and Collins (1985) determined that ethanol increases brain prostaglandin levels in a dose-, sex-, and genotype-dependent manner. These results were consistent with their hypothesis that part of ethanol's action may have to do with increases in brain prostaglandins. Gillian and Collins (1983) have also postulated that part of the effect of high doses of ethanol placed into the peritoneal cavity may stimulate peripheral production of prostaglandins or some other factor that then has CNS effects. This observation is consistent

with the findings of Deitrich et al. (1989) that the effects of phorbol esters are markedly different when administration is peripheral than when the compounds are administered ICV.

Additional Sources of Information

Previous Reviews and Conference Proceedings

Collins et al. (1976), Collins (1981, 1986), Deitrich and Spuhler (1984), Erwin (1986a), Erwin and McClearn (1981), Harris and Allan (1988). McClearn and Kakihana (1981), Palmer (1986), Smolen et al. (1984), Smolen and Collins (1986).

Other Studies

Alpern and McIntyre (1985b) (individual versus group learning), Church et al. (1977) (salsolinol), Deitrich et al. (1986) (ascorbic acid levels in brain), Dudek et al. (1984a) (apomorphine effects), Dudek and Fanelli (1980) (dopamine drugs), French et al. (submitted) (catecholamine), Gilliam and Collins (1982b, 1983c) (blood pH, PCO₂, and PO₂), Howerton and Collins (1984a) (norepinephrine release from brain slices), Horowitz et al. (1982) (dopamine β -hydroxylase), Morrow et al. (1988) (IP₃ binding in cerebellum), Reyes (1977) and Reyes et al. (1983) (gamma-glutamyl transpeptidase), Smolen (1980), Smolen and Collins (1984), and Smolen et al. (1984) (salsolinol), Towell and Erwin (1982) (glucose utilization in isolated perfused mouse brain), Zysset et al. (1983, 1985) (liver membranes), and Scheetz et al. (1987a,b) and Markham et al. (1987) (neuroanatomic changes during long-term ethanol administration).

References

- Allan, A.M.; Gallaher, E.J.; Gionet, S.E.; and Harris, R.A. Genetic selection for benzodiazepine ataxia produces functional changes in the gamma-aminobutyric acid receptor chloride channels complex. *Brain Res* 452:118-126, 1988a.
- Allan, A.M., and Harris, R.A. Gamma-amino butyric acid and alcohol actions: Neurochemical studies of long-sleep and short sleep mice. *Life Sci* 39:2005-2015, 1986.
- Allan, A.M., and Harris, R.A. Acute and chronic ethanol treatments alter GABA receptor-operated chloride channels. *Pharmacol Biochem Behav* 17:665-670, 1987.
- Allan, A.M., and Harris, R.A. Sensitivity to ethanol hypnosis and modulation of chloride channels does not co-segregate with pentobarbital sensitivity in HS mice. *Alcoholism: Clin Exp Res* 13:428-434, 1989.
- Allan, A.M.; Horowitz, G.P.; Isaacson, R.,L.; and Major, L.F. Adrenergic nervous system alteration and ethanol-induced narcosis in long-sleep and short-sleep mice. *Behav Neurol Biol* 42:134-139, 1984.
- Allan, A.M.; Huidobro-Toro, J.P.; Bleck, V.; and Harris, R.A. Alcohol and the GABA receptor-chloride channel complex of brain. *Alcohol Alcoholism Suppl* 1:643-646, 1987.
- Allan, A.M., and Isaacson, R.L. Ethanol-induced grooming in mice selectively bred for differential sensitivity to ethanol. *Behav Neurol Bio* 44:386-392, 1985.
- Allan, A.M.; Spuhler, K.P.; and Harris, R.A. Gamma-aminobutyric acid-activated chloride channels: Relationship to genetic differences in ethanol sensitivity. *J Pharmacol Exp Ther* 244:866-870, 1985b.
- Alpern, H.P., and Greer, C. A dopaminergic basis for the effects of amphetamine on a mouse "preadolescent hyperkinetic" model, *Life Sci* 21:93-98, 1977.

- Alpern, H.P., and McIntyre, T.D. Evidence that the selectively bred long- and short-sleep mouse lines display common narcotic reactions to many depressants. *Psychopharmacology* 85:456-459, 1985a.
- Alpern, H.P., and McIntyre, T.D. Individual vs. group learning development and social differences in two selected mouse lines. *Psychol Rec* 35:203-211, 1985b.
- Alpern, H.P., and McIntyre, T.D. Sedative hypnotic anomalies related to dose of pentobarbital in long-sleep and short-sleep selectively-bred mice. *Pharmacol Biochem Behav* 25:333-336, 1986.
- Baer, D.S., and Crumpacker, D.W. Fertility and offspring survival in mice selected for different sensitivities to alcohol. *Behav Genet* 7:95-103, 1977.
- Baizer, L.; Masserano, J.; and Weiner, N. Ethanol-induced changes in tyrosine hydroxylase activity in brains of mice selectively bred for differences in sensitivity to ethanol. *Pharmacol Biochem Behav* 15:945-949, 1981.
- Baker, R.C. Disassociation of cerebellar phospholipid composition and acute ethanol effects in mice selectively bred for differential sensitivity to ethanol. *Alcohol Drug Res* 7:291-299, 1987.
- Baker, R.; Melchior, C.; and Deitrich, R. The effect of halothane on mice selectively bred for differential sensitivity to alcohol. *Pharmacol Biochem Behav* 12:691-695, 1980.
- Baker, R.C.; Smolen, T., and Deitrich, R.A. Relationship between acute ethanol related responses in long-sleep and short-sleep mice. *Alcoholism Clin Exp Res* 11:574-578, 1987.
- Basile, A.; Hoffer, B.; and Dunwiddie, T. Differential sensitivity of cerebellar Purkinje neurons to ethanol in selectively outbred lines of mice: Maintenance in vitro independent of synaptic transmission. *Brain Res* 264:69-78, 1983.

- Brick, J., and Horowitz, G.P. Alcohol and morphine induced hypothermia in mice selected for sensitivity to ethanol. *Pharmacol Biochem Behav* 16:473-480, 1982.
- Brick, J., and Horowitz, G.P. Tolerance and cross-tolerance to morphine and ethanol in mice selectively bred for differential sensitivity to ethanol. *J Studies Alcohol* 44:770-779, 1983.
- Chan, A.W.K. Gamma aminobutyric acid in different strains of mice. Effect of ethanol. *Life Sci* 19:597-604, 1976.
- Church, A.C.; Fuller, J.L.; and Dann, L. Alcohol intake in selected lines of mice: Importance of sex and genotype. *J Comp Physiol Psychol* 93:242-246, 1979.
- Church, A.C.; Fuller, J.L.; and Dudek, B.C. Behavioral effects of salsolinol and ethanol on mice selected for sensitivity to alcohol-induced sleep time. *Drug Alcohol Depend* 2:443-452, 1977.
- Collins, A.C. A review of research using short-sleep and long-sleep mice. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. (ADM)81-1113. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 161-170.
- Collins, A.C. Genetics as a tool for identifying biological markers of drug abuse. In: Braude, M.C., and Chas, H.M., eds. *Genetic and Biologic Markers in Drug Abuse and Alcoholism*. DHHS Pub. No. ADM(86-144). Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1986. pp. 57-70.
- Collins, A.C.; Gilliam, D.M.; and Miner, L.L. Indomethacin pretreatment blocks the effects of high concentration of ethanol. *Alcoholism Clin Exp Res* 9:371-376, 1985.
- Collins, A.C.; Lebsack, M.E.; and Yeager, T.N. Mechanisms that underlie sex-linked and genotypically determined differences in the depressant actions of alcohol. *Ann NY Acad Sci* 273:303-316, 1976.

- Collins, A.C.; Smolen, A.; Waymon, A.L.; and Marks, M.J. Ethanol and temperature effects on five membrane bound enzymes. *Alcohol* 1:237-246, 1984.
- Daniell, L.C., and Harris, R.A. Alteration of ethanol and inositol trisphosphate-induced calcium release from brain and liver microsomes by chronic ethanol treatment and selective breeding for sensitivity to ethanol. (In press.)
- DeFiebre, C.M., and Collins, A.C. Decreased sensitivity to nicotine-induced seizures as a consequence of nicotine pretreatment in long-sleep and short-sleep mice. *Alcohol* 5:55-61, 1988.
- DeFiebre, C.M.; Medhurst, L.H.; and Collins, A.C. Nicotine response and nicotine receptors in long-sleep and short-sleep mice. *Alcohol* 4:483-501, 1987.
- DeFries, J.C.; Wilson, J.R.; Erwin, V.G.; and Petersen, D.R. LS x SS recombinant inbred strains of mice: Initial characterization. *Alcoholism Clin Exp Res* 13:196-200, 1989.
- Deitrich, R.A.; Bludeau, P.; and Baker, R.C. Brain ascorbic acid levels: Mice and rats selected for differences in acute reactions to ethanol. *Alcohol Alcoholism* 23:57-62, 1986.
- Deitrich, R.A.; Bludeau, P.; and Baker, R.C. Investigations of the role of protein kinase C in the acute sedative effects of alcohol. *Alcoholism Clin Exp Res*, in press.
- Deitrich, R.A., and Spuhler, K. Genetics of alcoholism and alcohol actions. In: Smart, R.G.; Cappell, H.D.; Glaser, F.B.; Israel, Y.; Kalant, H.; Popham, R.E.; Schmidt, W.; and Sellers, E.M. *Recent Advances in Alcohol and Drug Problems*. Vol. 8. New York: Plenum, 1984. pp. 47-98.
- Dibner, M.D.; Zahniser, N.R.; Wolfe, B.B.; Rabin R.A.; and Molinoff, P.B. Brain neurotransmitter receptor systems in mice genetically selected for differences to sensitivity to ethanol. *Pharmacol Biochem Behav* 12:509-514, 1980.

- Disbrow, J.K.; Masserano, T.M.; and Weiner, N. The thyroid status during postnatal maturation of the brain in mice genetically bred for differences in ethanol sensitivity. *J Pharmacol Exp Ther* 237:874-880, 1986.
- Disbrow, J.K., and Ruth, J.A. Differential glutamate release in brain regions of long-sleep and short-sleep mice. *Alcohol* 1:201-204, 1984.
- Disbrow, J.K., and Ruth, J.A. Differences in L-[3H] glutamate accumulation and endogenous L-glutamate content in synaptic vesicles from mice selectively bred for differences in ethanol sensitivity. *J Neurochem* 45:1294-1297, 1985.
- Dudek, B.C. Ethanol-induced conditioned taste aversions in mice that differ in neural sensitivity to ethanol. *J Studies Alcohol* 43:129-135, 1982.
- Dudek, B.C., and Abbott, M.E. A biometric genetic analysis of ethanol response in selectively bred long-sleep and short-sleep mice. *Behav Genet* 14:1-19, 1983.
- Dudek, B.C., and Abbott, M.E. The relationship between ethanol-induced locomotor activation and narcosis in long-sleep and short-sleep mice. *Alcoholism Clin Exp Res* 8:72-276, 1984.
- Dudek, B.C.; Abbott, M.E.; Garg, A.; and Phillips, T.J. Apomorphine effects on behavioral response to ethanol in mice selectively bred for differential sensitivity to ethanol. *Pharmacol Biochem Behav* 20:91-94, 1984a.
- Dudek, B.C.; Abbott, M.E.; and Phillips, T.J. Stimulant and depressant properties of sedative-hypnotics in mice selectively bred for differential sensitivity to ethanol. *Psychopharmacology* 82:46-51, 1984b.
- Dudek, B.C., and Fanelli, R.J. Effects of gamma-butyrolactone, amphetamine, and haloperidol in mice differing in sensitivity to alcohol. *Psychopharmacology* 68:89-97, 1980.
- Dudek, B.C., and Phillips, T.J. Locomotor stimulant and intoxicant properties of methanol, ethanol, tertiary butanol and pentobarbital in long-sleep and short-sleep mice. *Subst Alcohol Actions Misuse* 4:31-36, 1983.

- Erwin, V.G. Effects of neurotensin on thermoregulation, locomotor activity, and ethanol-induced sleep time in LS/Ibg and SS/Ibg mice. *Proc West Pharmacol Soc* 29:171-174, 1986a.
- Erwin, V.G. Genetic influences on acquisition of tolerance to alcohol. *Soc Biol* 32:222-228, 1986b.
- Erwin, V.G. Selective effects of cholinergic agonists and antagonists on ethanol sensitivity in LS and SS mice. *Proc West Pharmacol Soc* 130:353-356, 1987.
- Erwin, V.G., and Cornell, K. Effects of 6-hydroxydopamine on brain catecholamines and on acute actions of ethanol in LS/Ibg and SS/Ibg mice. *Alcoholism Clin Exp Res* 10:285-289, 1986.
- Erwin, V.G.; Heston, W.D.W.; McClearn, G.E.; and Deitrich, R.A. Effects of hypnotics on mice genetically selected for sensitivity to ethanol. *Pharmacol Biochem Behav* 4:679-683, 1976.
- Erwin, V.G., and Jones, B.C. Comparison of neurotensin levels, receptors and actions in LS/Ibg and SS/Ibg mice. *Peptides* (in press).
- Erwin, V.G., and Korte, A. Brain neurotensin receptors in mice bred for differences in sensitivity to ethanol. *Alcohol* 5:195-201, 1988.
- Erwin, V.G.; Korte, A.; and Jones, B.C. Central muscarinic cholinergic influences on ethanol sensitivity in long-sleep and short-sleep mice. *J Pharmacol Exp Ther* 247:857-862, 1988.
- Erwin, V.G.; Korte, A.; and Marty, M. Neurotensin selectively alters ethanol-induced anesthesia in LS/Ibg and SS/Ibg lines of mice. *Brain Res* 400:80-90, 1987.
- Erwin, V.G., and McClearn, G.E. Genetic influence on alcohol consumption and actions of alcohol. In: J. Galanter, ed. *Currents in Alcoholism* Vol. VII. New York: Grune & Stratton, 1981. pp. 405-420.

- Erwin, V.G.; McClearn, G.E.; and Kuse, A.R. Interrelationships of alcohol consumption, actions of alcohol and biochemical traits. *Pharmacol Biochem Behav* 13 (Suppl. 1):297-302, 1980.
- Erwin, V.G., and Su, N.W. Neurotensin and ethanol interactions on hypothermia and locomotor activity in LS and SS mice. *Alcoholism Clin Exp Res* 13:91-94, 1989.
- Erwin, V.C., and Towell, J.F. Ethanol-induced hyperglycemia mediated by the central nervous system. *Pharmacol Biochem Behav* 18:559-563, 1983.
- Fredholm, B.B.; Zahniser, M.R.; Weiner, G.R.; Proctor, W.R.; and Dunwiddie, T.V. Behavioral sensitivity to PIA in selectively bred mice is related to a number of A1 adenosine receptors but not to cyclic AMP accumulation in brain slices. *Eur J Pharmacol* 111:133-136, 1985.
- French, T.A.; Atkinson, N.; Petersen, D.R.; and Chung, L.W.K. Differential induction of hepatic microsomal ethanol and drug metabolism by 3-methylcholanthrene in "LS" and "SS" mice. *J Pharmacol Exp Ther* 209:404-410, 1979.
- French, T.A.; Clay, K.L.; Murphy, R.C.; and Weiner, N. Alpha-methyl para-tyrosine effects in mice selectively bred for differences in sensitivity to ethanol. *Biochem Pharmacol* 34:3811-3821, 1985a.
- French, T.A.; Clay, K.L.; and Weiner, N. The role of brain tyrosine availability in mediating differences in ethanol sensitivity in LS and SS mice. *J Pharmacol Expt Therap* 250:556-564, 1988b.
- French, T.A.; Masserano, J.M.; and Weiner, N. Ethanol-induced changes in tyrosine hydroxylase activity in adrenal glands of mice selectively bred for differences in sensitivity to ethanol. *J Pharmacol Exp Ther* 232:315-321, 1985b.
- French, T.A.; Masserano, J.M.; and Weiner, N. Further studies on the neurochemical mechanisms mediating differences in ethanol sensitivity in LS and SS mice. *Alcoholism Clin Exp Res* 12:215-235, 1988a.

- French, T.A., and Weiner, N. Effect of ethanol on tyrosine hydroxylation in brain regions of long- and short-sleep mice. *Alcohol* 1:247-252, 1984.
- Fuller, J.L. Regulation of ethanol intake in long- and short- sleep mice. In Erickson, K.; Sinclair, L.D.; and Kiianmaa, K.K. *Animal Models in Alcohol Research*. New York: Academic Press, 1980.
- George, F.R., and Collins, A.C. Ethanol's behavioral effects may be partly due to increases in brain prostaglandin production. *Alcoholism Clin Exp Res* 9:143-145, 1985.
- George, F.R.; Elmer, G.I.; and Collins, A.C. Indomethacin significantly reduces mortality due to acute ethanol overexposure. *Subst Alcohol Actions Misuse* 3:267-274, 1982.
- George, F.R.; Howerton, T.C.; Elmer, G.I.; and Collins, A.C. Antagonism of alcohol hypnosis by blockade of prostaglandin synthesis and activity: Genotype and time course effects. *Pharmacol Biochem Behav* 19:131-136, 1983.
- George, F.R.; Ritz, M.C.; Elmer, G.I.; and Collins, A.C. Time course of ethanol's effects on brain prostaglandins in LS and SS mice. *Life Sci* 39:1069-1075, 1986.
- Gilliam, D.M.; Bloedow, D.C.; and Collins, A.C. Nonlinear pharmacokinetics of ethanol elimination in long sleep and short sleep mice. *Alcoholism Clin Exp Res* 7:95-99, 1983.
- Gilliam, D.M., and Collins, A.C. Circadian and genetic effects on ethanol elimination in LS and SS mice. *Alcoholism Clin Exp Res* 6:344-348, 1982a.
- Gilliam, D.M., and Collins, A.C. Acute ethanol effects on blood pH, PCO₂ and PO₂ in LS and SS mice. *Physiol Behav* 28:879-883, 1982b.
- Gilliam, D.M., and Collins, A.C. Circadian and genetic influences on tissue sensitivity and sleep time to ethanol in LS and SS mice. *Pharmacol Biochem Behav* 18:803-808, 1983a.

- Gilliam, D.M., and Collins, A.C. Concentration-dependent effects of ethanol in LS and SS mice. *Alcoholism Clin Exp Res* 7:337-342, 1983b.
- Gilliam, D.M., and Collins, A.C. Differential effects of ethanol concentration on blood pH, PCO₂, and PO₂ in LS and SS mice. *Physiol Behav* 30:295-300, 1983c.
- Gilliam, D.M.; Phillips, T.J.; and Dudek, B.C. A comparison of ethanol absorption and narcosis in long- and short-sleep mice following intraperitoneal or intragastric ethanol administration. *Alcohol* 2:655-658, 1985.
- Gilliam, D.M.; Stilman, A.; Dudek, B.C.; and Riley, E.P. Fetal alcohol effects in long and short sleep mice: Activity, passive avoidance and in utero ethanol levels. *Neurobehav Toxicol Teratol* 9:349-357, 1987.
- Goldman, D.; Nelson, R.; Deitrich, R.A.; Baker, R.C.; Spuhler, K.; Markley, H.; Ebert, M.; and Merrill, C. Genetic brain polypeptide variants in inbred mice and in mouse strains with high and low sensitivity to alcohol. *Brain Res* 341:130-138, 1985.
- Goldstein, D.B.; Chin, J.H.; and Lyon, R.C. Ethanol disordering of spin-labeled mouse brain membranes: Correlation with genetically determined ethanol sensitivity of mice. *Proc Natl Acad Sci USA* 79:4231-4233, 1982.
- Goldstein, D.B., and Kakihana, R. Alcohol withdrawal reactions in mouse strains selectively bred for long or short sleep times. *Life Sci* 17:981-986, 1975.
- Greer, C.A., and Alpern, H.P. Mediation of myoclonic seizures by dopamine and clonic seizures by acetylcholine and GABA. *Life Sci* 21:385-392, 1977.
- Greer, C.A., and Alpern, H.P. Differential neurohumoral modulation of myoclonic and clonic seizures. *Arch Int Pharmacol* 236:74-85, 1978.
- Greer, C.A., and Alpern, H.P. Paradoxical effects of D-amphetamine upon seizure susceptibility in two selectively bred lines of mice. *Dev Psychobiol* 13:7-15, 1980.

- Harris, R.A., and Allan, A.M. Neurochemistry of brain chloride channels: Genetic variation in modulation by GABA agonists, alcohol and benzodiazepines. In: Biggio, G., and Costa, E. eds. *Chloride Channels and Their Modulation by Neurotransmitters and Drugs*. New York: Raven, 1988. pp. 189-197.
- Harris, R.A.; Burnett, R.; McQuilkin, S.; McClard, A.; and Simon F. Effect of ethanol on membrane order. *Ann NY Acad Sci* 492:125-135, 1987.
- Harris, R.A.; Zaccaro, L.M.; McQuilkin, S.; and McClard, A. Effects of ethanol and calcium on lipid order of membranes from mice selected for genetic differences in ethanol intoxication. *Alcohol* 5:251-257, 1988.
- Heston, W.D.; Erwin, V.G.; Anderson, S.M.; and Robbins, H. A comparison of the effects of alcohol on mice selectively bred for differences in ethanol sleep-time. *Life Sci* 14:365-370, 1974.
- Hjelle, J.J.; Atkinson, N.; and Petersen, D.R. The effects of chronic ethanol ingestion on ethanol binding to hepatic cytochrome P-450 and on certain hepatic and renal parameters in the "long sleep" and "short sleep" mouse. *Alcoholism Clin Exp Res* 5:198-203, 1981.
- Holmes, R.S.; Petersen, D.R.; and Deitrich, R.A. Biochemical genetic variants in mice selectively bred for sensitivity or resistance to ethanol-induced sedation. *Anim Genet* 17:235-244, 1986.
- Horowitz, G.P., and Allan, A. Morphine withdrawal in mice selectively bred for differential sensitivity to ethanol. *Pharmacol Biochem Behav* 16:35-39, 1982.
- Horowitz, G.P.; Dendel, P.S.; Allan, A.M.; and Major, L.F. Dopamine- β -hydroxylase activity and ethanol-induced sleep time in selectively bred and heterogeneous stock mice. *Behav Genet* 12:549-561, 1982.
- Howerton, T.C.; Burch, J.B.; O'Connor, M.F.; Miner, L.L.; and Collins, A.C. A genetic analysis of ethanol, pentobarbital, and methyprylon sleep-time response. *Alcoholism Clin Exp Res* 8:546-550, 1984.

- Howerton, T.C., and Collins, A.C. Ethanol-induced inhibition of GABA release from LS and SS mouse brain slices. *Alcohol* 1:471- 477, 1984a.
- Howerton, T.C., and Collins, A. Ethanol induced inhibition of norepinephrine release from brain slices obtained from LS and SS mice. *Alcohol* 1:47-53, 1984b.
- Howerton, T.C.; Marks, M.J.; and Collins, A.C. Norepinephrine, gamma-aminobutyric acid, and choline reuptake kinetics and the effects of ethanol in long-sleep and short-sleep mice. *Subst Alcohol Actions Misuse* 3:89-99, 1982a.
- Howerton, T.C.; O'Connor, M.F.; and Collins, A.C. Lipid solubility may control the hypnotic and hypothermic response of long-sleep and short-sleep mice to alcohols, barbiturates, methyprylon, and ethchlorvynol. *Alcoholism Clin Exp Res* 5:198-203, 1982b.
- Howerton, T.C.; O'Connor, M.F.; and Collins, A.C. Differential effects of long-chain alcohols in long- and short-sleep mice. *Psychopharmacology* 79:313-317, 1983a.
- Howerton, T.C.; O'Connor, M.F.; and Collins, A.C. Lipid solubility is correlated with hypotic and hypothermic responses of LS and SS mice to various depressant drugs. *J Pharmacol Exp Ther* 227:389-393, 1983b.
- Johnson, S.W.; Hoffer, B.J.; Baker, F.; and Freedman, R. Correlation of Purkinje neuron depression and hypnotic effects of ethanol in inbred strains of rats. *Alcoholism Clin Exp Res* 9:56-58, 1985.
- Kakihana, R. Endocrine and autonomic studies in mice selectively bred for different sensitivity to ethanol. In: Gross, M.M., ed. *Alcohol Intoxication and Withdrawal*. Vol. III. New York: Plenum Press, 1976a. pp. 83-95.
- Kakihana, R. Adrenocortical function in mice selectively bred for different sensitivity to ethanol. *Life Sci* 18:1131-1138, 1976b.
- Khanna, J.M.; Mayer, J.M.; Le, D.A.; and Kalant, H. Differential response to ethanol, pentobarbital and morphine in mice selectively bred for ethanol sensitivity. *Alcohol* 1:3-7, 1984.

- Koblin, D.D., and Deady, J.E. Anesthetic requirement in mice selectively bred for differences in ethanol sensitivity. *Anesthesiology* 53(Suppl. 3):552, 1980.
- Koblin, D.D., and Deady, J.E. Anesthetic requirement in mice selectively bred for differences in ethanol sensitivity. *Br J Anaesthesiol* 53:5-10, 1981.
- Markham, J.A.; Fifkova, E.; and Scheetz, A.J. Effect of chronic ethanol consumption on the fine structure of the dentate gyrus in long-sleep and short-sleep mice. *Exp Neurol* 95:290-302, 1987.
- Marks, M.J.; Smolen, A.; and Collins, A. Brain Na-K-ATPASES in mice differentially sensitive to alcohols. *Alcoholism Clin Exp Res* 8:390-396, 1984.
- Marley, R.J.; Freund, R.K.; and Wehner, J.M. Differential response to flurazepam in long-sleep and short-sleep mice. *Pharmacol Biochem Behav* (in press).
- Marley, R.J.; Miner, L.L.; Wehner, J.M.; and Collins, A.C. Differential effects of central nervous depressants in long-sleep and short-sleep mice. *J Pharmacol Exp Ther* 238:1028-1033, 1986.
- Marley, R.J.; Stinchcomb, A.; and Wehner, J.M. Further characterization of benzodiazepine receptor differences in long-sleep and short-sleep mice. *Life Sci* 43:1223-1231, 1988.
- Marley, R.J., and Wehner, J.M. Correlation between the enhancement of flunitrazepam binding by GABA and seizure susceptibility in mice. *Life Sci* 40:2215-2224, 1987.
- Marley, R.J., and Wehner, J.M. GABA enhancement of flunitrazepam binding in mice selectively bred for differential sensitivity to ethanol. *Life Sci* (in press).
- Martz, A.; Deitrich, R.A.; and Harris, R.A. Behavioral evidence for the involvement of gamma aminobutyric acid in the actions of ethanol. *Eur J Pharmacol* 89:53-62, 1983.

- Masserano, J.M., and Weiner, N. Investigations into the neurochemical mechanisms mediating differences in ethanol sensitivity in two lines of mice. *J Pharmacol Exp Ther* 221:404-409, 1982.
- McClearn, G.E., and Kakihana, R. Selective breeding for ethanol sensitivity: Short-sleep and long-sleep mice. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHSS Pub. No. (ADM)81- 1113. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 147-159.
- McIntyre, T.D., and Alpern, H.P. Reinterpretation of the literature indicates differential sensitivities of long-sleep and short-sleep mice are not specific to alcohol. *Psychopharmacology* 87:379-389, 1985.
- McIntyre, T.D., and Alpern, H.P. Thiopental, phenobarbital, and chlordiazepoxide induce the same differences in narcotic reaction as ethanol in long-sleep and short-sleep selectively bred mice. *Pharmacol Biochem Behav* 24:895-898, 1986a.
- McIntyre, T.D., and Alpern, H.P. GABAergic drugs can enhance or attenuate chlordinazepoxide-induced sleep time in a heterogeneous strain of mice. *Pharmacol Biochem Behav* 25:1077-1081, 1986b.
- McIntyre, T.D.; Trulles, R.; and Skolnick, P. Differences in the biophysical properties of benzodiazepine-gamma-aminobutyric acid receptor chloride channel complex in long-sleep and short-sleep mouse lines. *J Neurochem* 51:642-647, 1988.
- Miller, L.G.; Greenblatt, D.J.; Barnhill, J.G.; and Shader, R.I. Differential modulation of benzodiazepine receptor binding by ethanol in LS and SS mice. *Pharmacol Biochem Behav* 29:471-477, 1988.
- Moore, J., and Kakihana, R. Ethanol-induced hypothermia in mice: Influence of genotype on development of tolerance. *Life Sci* 23:2331-2338, 1978.

- Morrow, E.L., and Erwin, V.G. Calcium influence on neuronal sensitivity to ethanol in selectively bred mouse lines. *Pharmacol Biochem Behav* 24:949-954, 1986.
- Morrow, E.L., and Erwin, V.G. Calcium influence on neurotensin and β -endorphin enhancement of ethanol sensitivity in selectively bred mouse lines. *Alcohol Drug Res* 7:225-232, 1987.
- Morrow, E.L.; Erwin, V.G.; and Smith, T.L. {3H} inositol trisphosphate specific binding in cerebella of mouse lines selectively bred for differential ethanol sensitivity. *Proc West Pharmacol Soc* 31:269-271, 1988.
- O'Connor, M.F.; Howerton, T.C.; and Collins, A.C. Effects of pentobarbital in mice selected for differential sensitivity to ethanol. *Pharmacol Biochem Behav* 17:245-248, 1982.
- Palmer, M.R. Neurophysiological mechanisms in the genetics of ethanol sensitivity. *Soc Biol* 32:241-254, 1986.
- Palmer, M.R.; Basile, A.S.; Baker, R.; and Dunwiddie, T.V. Ethanol tolerance of cerebellar Purkinje neurons from selectively outbred mouse lines in vivo and in vitro electrophysiological investigations. *Alcoholism Clin Exp Res* 9:291-296, 1985.
- Palmer, M.R.; Morrow, E.L.; and Erwin, V.G. Calcium differentially alters behavioral and electrophysiological responses to ethanol in selectively bred mouse lines. *Alcoholism Clin Exp Res* 11:457-463, 1987a.
- Palmer, M.R.; Olson, L.; Dunwiddie, T.V.; Hoffer, B.J.; and Seiger, A. Neonatal cerebellectomy alters ethanol-induced sleep time of short-sleep but not long-sleep mice. *Pharmacol Biochem Behav* 20:153-9, 1984.
- Palmer, M.R.; Sorensen, S.M.; Freedman, R.; Olson, L.; Hoffer, B.; and Seiger, A. Differential ethanol sensitivity of intraocular cerebellar grafts in long-sleep and short-sleep mice. *J Pharmacol Exp Ther* 222:480-487, 1982.
- Palmer, M.R.; Wang, Y.; Fossom, L.; and Spuhler, K.P. Genetic correlation of ethanol-induced ataxia and cerebellar Purkinje neuron depression

- among inbred strains and selected lines of rats. *Alcoholism Clin Exp Res* 11:494-501, 1987b.
- Parsons, L.M.; Gallaher, E.J.; and Goldstein, D.B. Rapidly developing functional tolerance to ethanol is accompanied by increased erythrocyte cholesterol in mice. *J Pharmacol Exp Ther* 223:472-476, 1982.
- Pearlman, B.J., and Goldstein, D.B. Genetic influences on the central nervous system depressant and membrane disordering actions of ethanol and sodium valproate. *Mol Pharmacol* 26:547-552, 1984.
- Petersen, D.R., and Atkinson, N. Genetically mediated responses of microsomal ethanol oxidation in mice. *Adv Exp Med Biol* 132:117-128, 1979.
- Phillips, T.J.; Gilliam, D.M.; and Dudek, B.C. An evaluation of the role of ethanol clearance rate in the differential response of long-sleep and short-sleep mice to ethanol. *Alcohol* 1:373-378, 1984.
- Proctor, W.R.; Baker, R.C.; and Dunwiddie, T.V. Differential CNS sensitivity to PIA and theophylline in long-sleep and short-sleep mice. *Alcohol* 2:387-391, 1985.
- Proctor, W.R., and Dunwiddie, T.V. Behavioral sensitivity to purinergic drug parallels ethanol sensitivity in selectively bred mice. *Science* 224:519-521, 1984.
- Rabin, R.A., and Molinoff, P.B. Brain neurotransmitter receptor systems in alcohol treated mice and in mice genetically selected for differences in sensitivity to alcohol. In: Thurman, R.G., ed. *Alcohol and Aldehyde Metabolizing Systems*. Vol. IV. New York: Plenum Press, 1979. pp. 787-794.
- Reyes, E. Brain-glutamyl transpeptidase levels in mice genetically selected for sensitivity to ethanol. *Res Commun Chem Pathol Pharmacol* 17:353-356, 1977.

- Reyes, E.; Rivera, J.M.; and Murray, T. Isolation and partial characterization of gamma-glutamyl transpeptidase from mice differing in alcohol sensitivity. *Neurobehav Toxicol Teratol* 5:189-193, 1983.
- Romm, E., and Collins, A.C. Body temperature influences on ethanol elimination rate. *Alcohol* 4:189-198, 1987.
- Ryan, L.J.; Barr, J.E.; Sanders, B.; and Sharpless, S.K. Electro-physiological responses to ethanol, pentobarbital, and nicotine in mice genetically selected for differential sensitivity to ethanol. *J Comp Physiol Psychol* 93:1035-1052, 1979.
- Sanders, B. Sensitivity to low doses of ethanol and pentobarbital in mice selected for sensitivity to hypnotic doses of ethanol. *J Comp Physiol Psychol* 90:394-398, 1975.
- Sanders, B. Withdrawal-like signs induced by a single administration of ethanol in mice that differ in ethanol sensitivity. *Psychopharmacology* 68:109-113, 1980.
- Sanders, B., and Sharpless, S.K. Dissociation between the anticonvulsant action of alcohol and its depressant action in mice of different genotypes. *Life Sci* 23:2593-2600, 1978.
- Sanders, B.; Sharpless, S.K.; Collins, A.C.; McClearn, G.E.; and Flanagan, C. Activating and anesthetic effects of general depressants. *Psychopharmacology* 56:185-198, 1978.
- Scheetz, A.J.; Markham, J.A.; and Fifkova, E. Changes in the frequency of basket cells in the dentate fascia following chronic ethanol administration in mice. *Brain Res* 403:151-154, 1987a.
- Scheetz, A.J.; Markham, J.A.; and Fifkova, E. The effect of chronic ethanol consumption on the fine structure of the CA1 stratum oriens in short-sleep and long-sleep mice: Short-term and long-term exposure. *Brain Res* 409:329-334, 1987b.

- Seiger, A.; Sorensen, S.M.; and Palmer, M.R. Cerebellar role in the differential ethanol sensitivity of long sleep and short sleep mice. *Pharmacol Biochem Behav* 18:495-499, 1983.
- Siemens, A.J., and Chan, A.W.K. Differential effects of pentobarbital and ethanol in mice. *Life Sci* 19:581-590, 1976.
- Smolen, A.; Marks, M.J.; Smolen, T.N.; and Collins, A.C. Dose and route of administration alter the relative elimination of ethanol by long-sleep and short-sleep mice. *Alcoholism Clin Exp Res* 10:198-204, 1986.
- Smolen, A., and Smolen, T.N. Genetic influence on increase seizure susceptibility in pregnancy. *Life Sci* 39:1525-1530, 1986.
- Smolen, A., and Smolen, T.N. Demonstration of a threshold concentration for ethanol at the time of regaining the righting response in long-sleep and short-sleep mice. *Alcohol Drug Res* 7:279-283.
- Smolen, A., and Smolen, T.N. Reproducibility of ethanol eliminates rates in long-sleep and short-sleep mice. (Submitted.)
- Smolen, A.; Smolen, T.N.; and Collins, A. The influence of age, sex, and genotype on the subcellular distribution of hepatic aldehyde dehydrogenase activity in the mouse. *Comp Biochem Physiol* 73:815-822, 1982.
- Smolen, A.; Smolen, T.N.; and VandeKamp, J.C. Sensitivity of inbred and selectively bred mice to ethanol. *Alcohol* 4:57-62, 1987.
- Smolen, T.N. Ethanol-salsolinol interactions in long-sleep and short-sleep mice. *Behav Genet* 10:497-498, 1980.
- Smolen, T.N., and Collins, A.C. Behavioral effects of ethanol and salsolinol in mice selectively bred for acute sensitivity to ethanol. *Pharmacol Biochem Behav* 20:281-287, 1984.
- Smolen, T.N., and Collins, A.C. Neurochemical mechanisms in the genetics of alcohol phenotypes. *Soc Biol* 32:255-271, 1986.

- Smolen, T.N.; Howerton, T.C.; and Collins, A.C. Effects of ethanol and salsolinol on catecholamine function in LS and SS mice. *Pharmacol Biochem Behav* 20:125-131, 1984.
- Smolen, T.N., and Smolen, A. Blood and brain ethanol concentrations during absorption and distribution in long-sleep and short-sleep mice. (Submitted.)
- Smolen, T.N.; Smolen, A.; and VandeKamp, J.C. Developmental profile of alcohol and aldehyde dehydrogenase in long-sleep and short-sleep mice. *Ann NY Acad Sci* 492:66-68, 1986.
- Sorensen, S.; Dunwiddie, T.; McClearn, G.; Freedman, R.; and Hoffer, B. Ethanol-induced depressions in cerebellar and hippocampal neurons of mice selectively bred for differences in ethanol sensitivity: An electrophysiological study. *Pharmacol Biochem Behav* 14:227-234, 1981.
- Sorensen, S.; Palmer, M.R.; Dunwiddie, T.; and Hoffer, B. Electrophysiological correlates of ethanol-induced sedation in differentially sensitive line of mice. *Science* 210:1143-1145, 1980.
- Spuhler, K., and Deitrich, R.A. Correlative analysis of ethanol-related phenotypes in rat inbred strains. *Pharmacol Biochem Behav* 8:480-484, 1984.
- Spuhler, K.; Gerhardt, G.; and Palmer, M. Effect of central nervous system noradrenergic lesion by DSP-4 N-2 chloroethyl-N-ethyl-2-bromobenzylamine on ethanol sleeptime in long-sleep and short-sleep mice. *Alcohol* 4:419-424, 1987.
- Spuhler, K.; Hoffer, B.; Weiner, N.; and Palmer, M. Evidence for genetic correlation of hypnotic effects and cerebellar Purkinje neuron depression in response to ethanol in mice. *Pharmacol Biochem Behav* 17:569-578, 1982.
- Stinchcomb, A.; Bowers, B.J.; and Wehner, J.M. The effects of ethanol and Ro 15-4513 on elevated plus maze and rotorod performance in long-sleep and short-sleep mice. *Alcohol*, in press.

- Swanberg, K.M. Relationships between adrenal corticosteroids and alcohol tolerance: The effects of neonatal exposure to alcohol on mice selectively bred for different sensitivities to alcohol. *Dissert Abst Int* 37(12-B, Pt. 2):6397, 1977.
- Swanberg, K.M., and Wilson, J.R. Genetic and ethanol-related differences in maternal behavior and offspring viability in mice. *Dev Psychobiol* 12:610-66, 1979.
- Swanberg, K.M.; Wilson, J.R.; and Kalisker, A. Developmental and genotypic effects on pituitary adrenal function and alcohol tolerance in mice. *Dev Psychobiol* 12:201-210, 1979.
- Tabakoff, B., and Ritzmann, R.F. Acute tolerance in inbred and selected lines of mice. *Drug Alcohol Depend* 4:87-90, 1979.
- Tabakoff, B.; Ritzmann, R.; Raju, T.S.; and Deitrich, R.A. Characterization of acute and chronic tolerance in mice selected for inherent differences in sensitivity to ethanol. *Alcoholism Clin Exp Res* 4:70-73, 1980.
- Towell, J.R., III, and Erwin, V.C. Effects of ethanol and temperature on glucose utilization in the in vivo and isolated perfused mouse brain. *Alcoholism Clin Exp Res* 6:110-116, 1982.
- Ullman, M.D.; Baker, R.C.; and Deitrich, R.A. Gangliosides of LS and SS mouse cerebellum and cerebellar synaptosomal plasma membranes. *Alcoholism Clin Exp Res* 11:158-162, 1987.
- Weiner, N.; Disbrow, J.K.; French, T.A.; and Masserano, J.M. The influence of catecholamine systems and thyroid function on the actions of ethanol in long-sleep (LS) and short-sleep (SS) mice. *Ann NY Acad Sci* 492:375-383, 1987.
- Zgombick, J.M., and Erwin, V.G. Central mechanisms of ethanol-induced adrenocortical response in selectively bred lines of mice. *Neuroendocrinology* 46:324-332, 1987.

Zgombick, J.M.; Erwin, V.C.; and Cornell, K. Ethanol-induced adrenomedullary catecholamine secretion in LS/Ibg and SS/Ibg mice. *J Pharmacol Exp Ther* 236:634-640, 1986.

Zysset, T.; Polokoff, M.; Warren, G.H.; and Simon, F.R. Effect of chronic ethanol administration on enzyme and lipid properties of liver plasma membranes in long-sleep and short-sleep mice. *Hepatology* 5:531-537, 1985.

Zysset, T.; Sutherland, E.; and Simon, F.R. Studies on the differences in Na-K-ATPase and lipid properties of liver plasma membranes in long-sleep and short-sleep mice. *Alcoholism Clin Exp Res* 7:85-92, 1983.

Finnish Selective Breeding Studies for Initial Sensitivity to Ethanol: Update 1988 on the AT and ANT Rat Lines

*C. J. Peter Eriksson*¹

Introduction

Ethanol produces a variety of acute physiologic-behavioral effects that together constitute intoxication. Over the past decades, it has become clear that the initial sensitivity to at least some of these effects is strongly influenced by genetic factors (National Institute on Alcohol Abuse and Alcoholism 1981). This has been demonstrated in selection studies (Riley et al. 1977; Rusi et al. 1977; McClearn and Anderson 1979; Crabbe et al. 1987*a,b*) and strain comparisons (Belknap 1980; Crabbe et al. 1982; Crabbe 1983) with experimental animals and in human twin (Propping 1977*a,b*) and population (Goedde et al. 1983) studies.

In our laboratory, two rat lines have been developed by selective outbreeding for high and low degrees of ethanol-induced motor impairment as measured by the tilting-plane and rotarod tests (Rusi et al. 1977; Eriksson and Rusi

¹Research Laboratories for the Finnish State Alcohol Company, Alko Ltd. POB 350, SF-00101 Helsinki, Finland.

1981). This overview summarizes our current knowledge on these AT (alcohol-tolerant) and ANT (alcohol-nontolerant) rat lines.

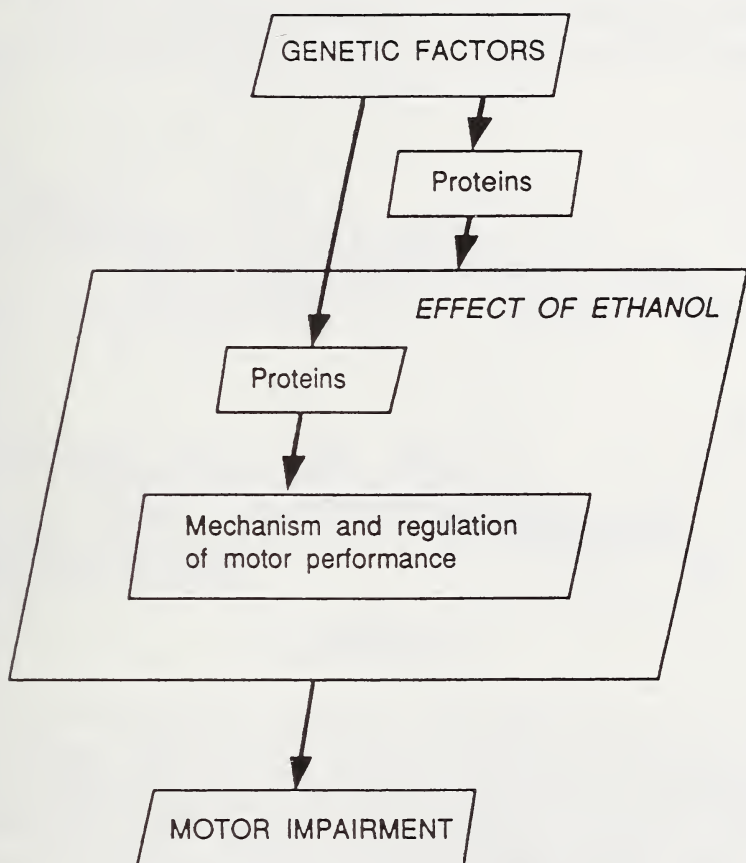
General Aspects

Motor performance, like many other behaviors, is regulated by a network of complex reactions that may involve both central and peripheral mechanisms. Each of these reactions may, of course, include biologic variation, part of which has a genetic origin. The biologic variation, especially at the site of the ethanol action, will naturally be reflected in the variation of the effects by this drug. Thus, genetically determined variation in the normal regulation of motor performance will, unless it is specifically excluded, be included in the selection for differential ethanol effects on motor performance. In addition, such a selection may include all of the genetic variation in the ethanol-initiated network of reactions before entering the final pathway of reactions regulating the motor performance. The different levels of genetic variability are schematized in figure 1.

One of the difficulties in the design of a selection procedure for ethanol-induced motor impairment is the decision of which selection parameter should be included or excluded. For example, if emphasis were on keeping the basal performance constant, more selection pressure would be directed into the "own effects" of ethanol. In this case, however, there would be the risk that one would lose an important source of genetic variability that perhaps appears at the site of the motor pathway that ethanol affects.

Our early selection procedures with the AT and ANT rats involved the tilting-plane test in combination with the open-field test for the first two generations and with the rotarod test for the next 12 generations, as described earlier (Eriksson and Rusi 1981). The breeding animals were, however, primarily selected on the basis of their performance on the tilting plane, in which the difference in sliding angle before and 30 min after an ethanol injection of 2 g/kg of body weight was recorded. From F_{15} on, only tilting-plane tests were performed. Development of the different lines is depicted in figure 2 (which shows original unpublished results provided by M.

Figure 1.—Genetic factors in ethanol-induced motor impairment

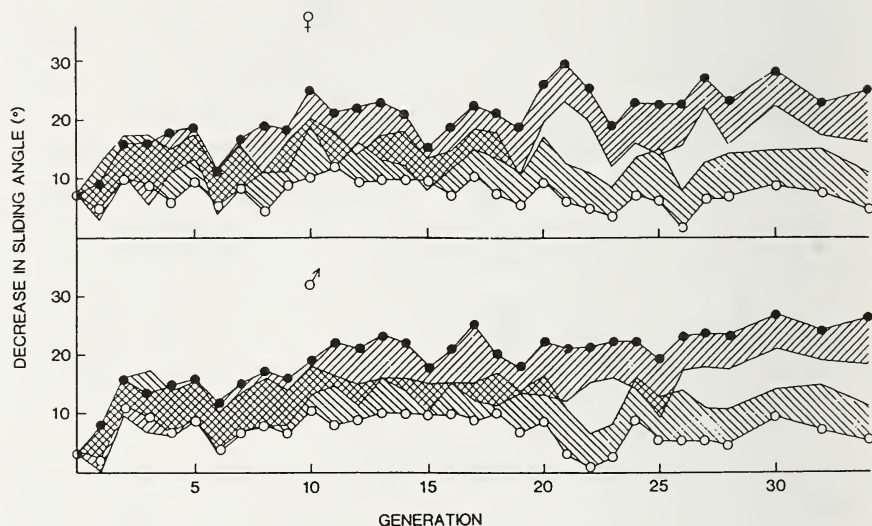


Sarviharju, P. Hyytiä, and K. Tuominen). As can be seen, the selection has mainly concerned the ANT line.

To avoid selection pressure on the pharmacokinetics of ethanol, we selected only rats with the same ethanol concentration at the end of the tilting-plane tests. Consequently, no blood ethanol line differences have appeared, the ethanol concentration ranging between 40 and 50 mM right after the tests.

In the early selection, where no emphasis was put on the starting performance, line differences, with the ANT animals starting from a higher

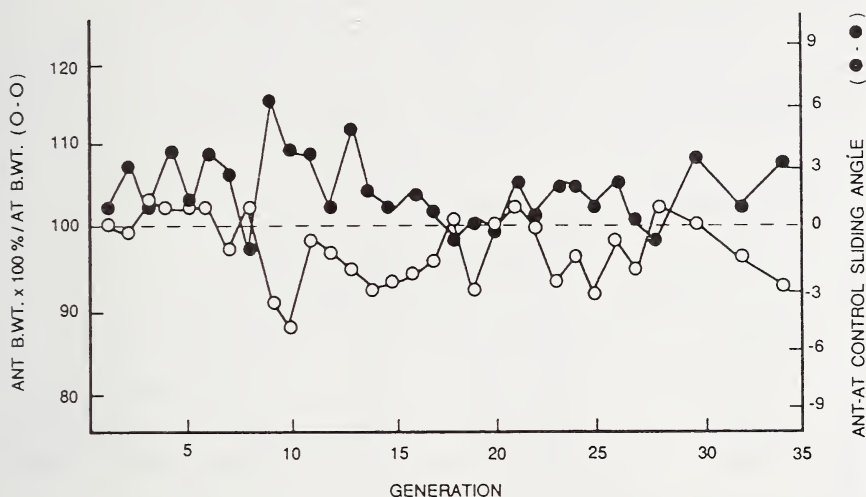
Figure 2.—The effect of selective breeding in separating AT and ANT rat lines. AT means (o) plus standard deviations; ANT means (●) minus standard deviations. ($n=12-49$)



performance (steeper angle), emerged (figure 3, which depicts original unpublished results provided by M. Sarviharju, P. Hyytiä, and K. Tuominen). In later generations, selection pressure was applied every now and then to keep the baseline performances equal, which resulted in subsequent disappearance of the differences.

Another "base" difference, which has been observed from time to time, has been body weight, with the ANT animals being lighter than the AT animals (figure 3). As can be observed in the figure, these body weight differences correlated negatively to the basal performance values. A similar observation was made with female animals (results not shown). As a result of these relationships, future selection of our AT and ANT lines will require equal body weights and initial performances as selection criteria.

Figure 3.—Development of AT-ANT line differences in percent of body weight and baseline motor performance; means of male rats ($n=21-49$)



Behavioral Aspects

Motor Impairment

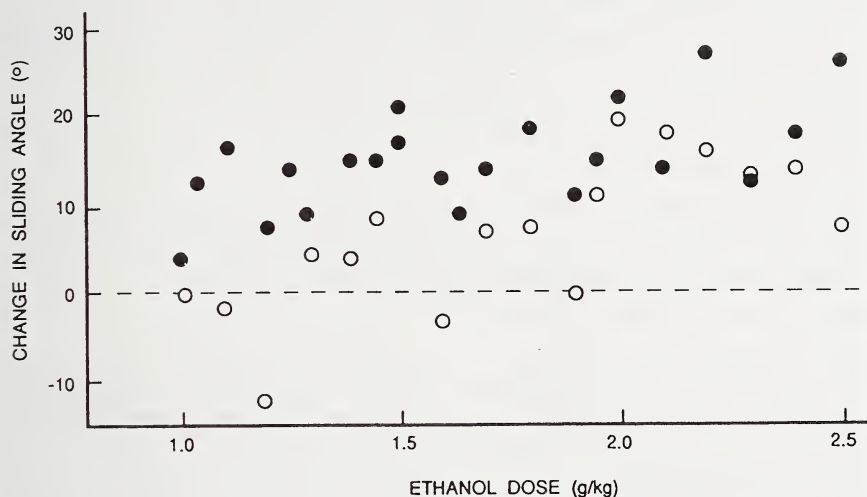
Table 1 lists the behavioral comparisons made between the AT and ANT lines. As can be observed, other ethanol doses were also tested in F_{12} with regard to the tilting-plane test, which demonstrated that the line differences held throughout a wide range of ethanol doses (see figure 4 for original data). In a later generation (F_{15}), a low dose of ethanol was tested, with similar results (Sinclair et al. 1982). These line differences were also replicated with another motor test, involving a horizontal wire (Bonetti et al. 1982), in generations F_{29-30} , and in a test using the moderate ethanol dose of 1.4 g/kg (Hellevuo and Korpi 1988). However, no line difference was observed when the high dose of 2.75 g/kg was used in the tilting-plane test. Perhaps this dose produced some kind of stronger ataxia that did not share a common genetic background with the factors involved in the normal tilting-plane motor impairment. Another possibility is that a ceiling effect simply prevented a line difference from being seen. A similar tendency can be seen in figure 4 at doses above 2 g/kg.

Table 1.—Behavioral comparisons of the ANT and AT rat lines

Parameter	Difference	F	Reference
Motor impairment by ethanol			
2 g/kg	ANT > AT	1-34	Figure 1
1.0-2.5 g/kg	ANT > AT	12	Figure 4
Acquired tolerance	ANT < AT (NS) ^a	12	Eriksson et al. (1982)
1.0 g/kg	ANT > AT	15	Sinclair et al. (1982)
Acute tolerance	ANT < AT	28	Le and Kiianmaa (1989)
1.4 g/kg	ANT > AT	29-30	Hellevuo and Korpi (1988)
2.75 g/kg	NS	29-30	Hellevuo and Korpi (1988)
Voluntary ethanol consumption			
Free choice	ANT < AT	7	Eriksson and Rusi (1981)
Forced	NS		
Free after forced	ANT < AT (NS)		
Ethanol narcosis			
4 g/kg	ANT > AT (NS)	12	Eriksson et al. (1982)
2.0-6.0 g/kg	NS	13	Eriksson and Sarviharju (1984)
3.5 g/kg	ANT > AT	28	Le and Kiianmaa (1989)
Acquired tolerance	ANT < AT	28	Le and Kiianmaa (1989)
Ethanol hypothermia			
4 g/kg	ANT > AT (NS)	13	Eriksson and Sarviharju (1984)
2.5-3.5 g/kg	NS	28	Le and Kiianmaa (1989)
Acute tolerance	NS	28	Le and Kiianmaa (1989)
Acquired tolerance	NS	28	Le and Kiianmaa (1989)
Swim activity	ANT < AT	27	Sinclair et al. (1987)

^aNS, not significant.

Figure 4.—Effects of different ethanol doses on motor performance; individual values for AT (o) and ANT (●) male rats



Other Components of Ethanol Intoxication

An interesting question is to what extent the various components of ethanol intoxication, such as different kinds of motor impairment, activity changes, hypothermia, and narcosis, involve common reactions influenced by common genetic factors. In generations F_{12-13} and F_{28} , the effects of ethanol on body temperature and loss of righting reflex were tested (table 1). Little or no line difference regarding hypothermia and sleep time was observed, and it was concluded that the genetically determined mechanisms influencing motor impairment are for the most part dissociated from factors determining the hypothermic and narcotic effects of ethanol (Eriksson and Sarviharju 1984). Similar conclusions have also been on the basis of strain (Crabbe 1983) or individual (Eriksson et al. 1982) correlations.

Acute and Acquired Tolerance

Another question of relevance is whether the genetically influenced mechanisms regulating initial sensitivity, acute and acquired tolerance, are common or separate. Support for such relationships was observed in generation F_{12} (Eriksson et al. 1982), in which the development of acquired tolerance tended to be more rapid in the AT rats (table 1). The tendency was not very substantial, and it was concluded that the primary mechanisms regulating initial motor impairment were genetically distinct from those determining acquired tolerance to this component of ethanol intoxication. Similarly, with regard to ethanol-induced motor impairment, Bass and Lester (1980) found no relationship between initial sensitivity and acquired tolerance in their MA and LA rat lines.

The existence of some common regulative factors cannot, of course, be excluded. Indeed, evidence that part of the line differences regarding motor impairment could be due to differential development of acute tolerance was observed in generation F_{28} (Le and Kiianmaa 1989). In this generation, it was also observed that subacute tolerance to the duration of loss of righting reflex was somewhat higher in the AT than in the ANT line (table 1). Similarly, by comparing the acquired sleep time tolerance in LS and SS mice, faster development of tolerance was observed in the SS line (Tabakoff et al. 1980).

Voluntary Ethanol Consumption

In previous selection studies, it was observed that animals that had been genetically prone to low drinking were more sensitive to a variety of components of ethanol intoxication, including motor impairment (Nikander and Pekkanen 1977; Rusi et al. 1977; Malila 1978; Li et al. 1979, 1981). Ethanol intoxication has, in fact, been suggested as one of the major factors involved in the regulation of voluntary ethanol intake in experimental animals (Wallgren and Barry 1970; Eriksson 1981).

Voluntary ethanol consumption has not been thoroughly investigated in the AT and ANT rat lines. Only one study (Eriksson and Rusi 1981) from the F_7 generation documents that the ANT rats consume less ethanol in a free-choice situation than do the AT animals. Whether this result holds in later

generations and whether this relationship is meaningful remain to be determined. Also, whether it is the high ethanol sensitivity limiting the consumption or whether less sensitivity is somehow associated with more motivation for ethanol drinking is a challenging question.

Basic (Sober) Behaviors

As discussed above, every now and then the AT and ANT rats differ with regard to their basic (control) motor performances on the tilting plane (figure 3). The lines also differ with regard to other sober behaviors. Thus, the ANT rats generally appear to be "calmer," "less nervous," or simply "less active" than ATs (Sinclair et al. 1987). This finding was quantified by measuring the active time in the low-stress forced-swim test, in which the ANT rats displayed less activity than the AT animals (Sinclair et al. 1987; table 1). The relevance of this finding is unclear, because swim test results were not found to correlate to tilting-plane results in an unselected Long Evans population (Sinclair et al. 1987).

It has also been noted that line differences in hair color have developed over the years, and computer simulations indicated that genetic drift could easily have produced differences in the current lines that are unrelated to ethanol sensitivity (Sinclair et al. 1987).

Neurochemical Aspects

Virtually every neurochemical system investigated seems to be affected by or associated with ethanol intoxication. The specific mechanisms for any of the components of ethanol intoxication are, however, as yet unsolved. The AT and ANT rat lines, with the differential motor impairments, as well as any other animal line selected for some other component of ethanol intoxication provide an excellent tool for resolving which neurochemical mechanisms are involved at least in the genetically determined sensitivity to ethanol. In the following section, I will outline our neurochemical data.

Opiate Systems

In generation F₁₅, the AT and ANT rats were screened for both ethanol- and morphine-induced motor impairment (Sinclair et al. 1982). Morphine did not differentially affect the rat lines, despite a huge line difference in the effect of ethanol, which supported the notion of independence of ethanol-induced motor impairment from opiate systems. However, when the effect of naloxone on the motor impairment by a low dose of ethanol was tested (Sinclair et al. 1982), an increase in impairment was observed in the AT rats. Presuming that naloxone inhibits the "stimulatory" effects of ethanol (Middaugh et al. 1978), it is possible that such a stimulation could counteract motor impairment. Thus, the opiate system could indirectly be involved in the modulation of the motor impairment line differences, with the AT rats having inherited more of the activation systems. The effect, at least proportionally, seems to be of little importance at higher ethanol concentrations.

GABA-Benzodiazepine Systems

The γ -aminobutyric acid benzodiazepine system has in recent studies been suggested as a likely neurochemical target for the different components of ethanol intoxication (McIntyre and Alpern 1985; Ticku and Kulkarni 1988). An early indication of GABAergic mechanisms in our AT-ANT line differences (F₁₅) was the observation by Sinclair et al. (1982), according to which barbital differentially impaired motor performance (table 2). More comprehensively, it was later (F₂₄) observed that, in addition to barbital, the benzodiazepine lorazepam impaired motor performance more in ANT than in AT rats (Hellevuo et al. 1987; table 2). It was concluded that ethanol-induced motor impairment has mechanisms in common with impairment by barbiturates and benzodiazepines and that the commonality involves the GABA-benzodiazepine system. These findings are in line with those of other selection studies, in which ethanol-induced loss of righting reflex also has been associated with the GABA-benzodiazepine system (McIntyre and Alpern 1985).

In an attempt to further localize and characterize postsynaptic molecular GABA mechanisms, in vitro ligand-binding studies were performed to

Table 2.—GABA-benzodiazepine comparisons of the ANT and AT rat lines

Parameter	Difference	F	Reference
Motor impairment by barbital	ANT>AT	15	Sinclair et al. (1982)
Motor impairment by barbital and lorazepam	ANT>AT	24	Hellevuo et al. (1987)
Muscimol and flunitrazepam binding in detergent-treated cortex and hippocampus membranes and in cerebellum but in buffer-washed membranes:	ANT>AT	25-26	Malminen and Korpi (1988)
cortex and hippocampus, cerebellum (muscimol), (flunitrazepam)	NS ANT<AT ANT>AT		
Picrotoxin antagonism of motor impairment by barbital	ANT>AT	28	Kiianmaa et al. (1988)
Effect of Ro 15-4513 on motor impairment by ethanol	NS	29-30	Hellevuo and Korpi (1988)
GABA turnover: rate in cerebellum, level in striatum, ethanol-induced inhibition in cortex	ANT>AT ANT>AT ANT<AT	32	Kiianmaa et al. (1988)

^aNS, not significant.

compare GABA-benzodiazepine receptor-chloride ionophore complexes in various brain regions of the AT and ANT rats (Malminen and Korpi 1988). Several significant but fairly small line differences in the binding parameters of the GABA agonist muscimol and the benzodiazepine agonist flunitrazepam were observed (table 2). The authors concluded that the difference in ethanol sensitivity between the AT and ANT rat lines could not be explained by a differential binding function of the GABA-benzodiazepine receptor-chloride ionophore complex.

In a later line comparison ($F_{29,30}$), the effect of Ro 15-4513, a partial inverse agonist of the benzodiazepine receptor, on ethanol-induced motor impairment was tested (Hellevuo and Korpi 1988). No effect on ethanol-induced impairment was observed in either line of rats (table 2) even though Ro 15-4513 completely abolished lorazepam-induced motor impairment in an unselected population of Wistar rats. This finding suggests that the benzodiazepine recognition site may not be the primary source for the genetic variability in these rat lines. However, further studies have revealed a variety of line differences in GABA turnover (table 2), which suggests line differences in the sensitivity of the GABA neurons to ethanol (review in Kiianmaa et al. 1988).

Monoamine Systems

The catecholamine (CA) and serotonin (5-HT) transmitter systems have been classical targets for investigations on the neuronal mechanisms of ethanol intoxication (reviewed in Hunt and Majchrowicz 1979; Tabakoff and Hoffman 1980; Pohorecky and Brick 1988). Our AT and ANT monoamine studies (summarized in table 3) started in generation $F_{5,6}$ (Ahtee et al. 1980). In this study, higher concentrations of dopamine (DA) and noradrenaline (NA) were observed in the limbic regions of the ANT rats than in those of the ATs. Higher basal DA levels in the ANT brains have also been observed in later generations (F_{18} [Kiianmaa and Tabakoff 1984]; F_{26} [Kiianmaa et al. 1988]). Slightly higher brain dopa decarboxylase activities have been observed in this line as well (Pispa et al. 1986). Originally, no differences in catabolism of DA were found (Ahtee et al. 1980), which seemed to contradict later observations of faster DA catabolism in the ANT than in AT rats (Kiianmaa and Tabakoff 1984; Kiianmaa et al. 1988). However, recalculation

Table 3.—Monoamine comparisons of the ANT and AT rat lines

Parameter	Difference	F	Reference
DA			
Limbic levels	ANT>AT	5-6	Ahtee et al. (1980)
Catabolism	NS ^a		
Striatum levels	NS		
Catabolism	NS		
NA			
Limbic levels	ANT>AT		
Catabolism	NS		
Brain stem levels	NS		
Catabolism	ANT>AT		
Other parts: levels and catabolism	NS		
DA			
Striatum levels and metabolism	ANT>AT	18	Kiianmaa and Tabakoff (1984)
Increase by ethanol	NS		
NA			
Cortex levels (basal or after ethanol)	NS		
HT			
Brain levels, synthesis, and catabolism (basal or after ethanol)	NS	20-22	Kiianmaa (1984)
HT			
Frontal cortex level elevation by lorazepam, ethanol or barbital	ANT<AT	24	Hellevuo and Kiianmaa (1988)
Striatum elevation by lorazepam	ANT>AT		

^aNS, not significant.

Table 3.—Monoamine comparisons of the ANT and AT rat lines
Continued

Parameter	Difference	F	Reference
HT (Cont.)			
Lower brain stem elevation by barbital	ANT < AT		
Other parts and drugs	NS ^a		
Synthesis, catabolism	NS		
DA			
Limbic level elevation by lorazepam	ANT < AT		
Other parts and drugs	NS		
Synthesis	NS		
Limbic catabolism: decrease by barbital	ANT > AT		
Other parts and drugs	NS		
NA			
Frontal cortex level elevation by lorazepam	ANT > AT		
Other parts and drugs	NS		
DA			
Striatum D2 receptors: K_d and B_{max}	NS	25	Korpi et al. (1987 ^{a,b})
Limbic levels and catabolism	ANT > AT	26	Kiianmaa et al. (1988)
NA			
Cerebellar levels	ANT < AT		

^aNS, not significant.

of the early data from $F_{5,6}$ demonstrates that indeed, already at that point of selection, there was about 30 percent faster DA catabolism in the ANT than in the AT rats. Ethanol has been observed to activate DA metabolism in a number of brain parts, but no line differences appeared in this effect (Kiianmaa and Tabakoff 1984; Hellevuo and Kiianmaa 1988). These DA mechanisms seem not to be related to those of the barbiturate and benzodiazepine mechanisms, since these drugs affected the AT and ANT DA systems differently than did ethanol (Hellevuo and Kiianmaa 1988). Neither do the DA mechanisms seem related to the D2 receptor parameters, as shown in F_{25} rats (Korpi et al. 1987*a,b*). Thus, it has been suggested that the catecholaminergic system is not involved in production of the genetically determined line differences in ethanol-induced motor impairment (Kiianmaa et al. 1988). However, the relevance of the line differences regarding DA metabolism remains to be determined.

Similar to the original line difference showing higher NA levels in the limbic regions of the ANT than of AT rats ($F_{5,6}$), higher NA levels have also been determined in the frontal cortex of the ANT rats of F_{24} (Hellevuo and Kiianmaa 1988). In the cerebellum, however, opposite differences have been observed (F_{26} [Kiianmaa et al. 1988]). The relevance of these differences also remains to be determined.

Few studies have dealt with the serotonin (5-HT) systems of the AT and ANT rats. No basal differences regarding concentrations, synthesis, and catabolism have been observed in the brains of the rat lines (Kiianmaa 1984). However, ethanol has been observed to lower 5-HT concentrations in the frontal cortex of ANT rats and to elevate the corresponding levels in AT rats (Hellevuo and Kiianmaa 1988). Similar differential effects appeared also with barbital or lorazepam treatment, suggesting that these drugs could have a commonality regarding the mechanisms of motor impairment.

Acetylcholine Systems

The involvement of cholinergic systems in the mechanisms of motor impairment by ethanol in the AT and ANT rat lines was first investigated in

generation F_{20} (Eriksson and Guerri 1986). Cerebral and cerebellar acetylcholinesterase activities were measured and marked line differences were observed, with the cerebral activities being higher in the ANT rats than in the ATs. Ethanol injections had no effect on these basal activities. In the next series of experiments, the cerebral distribution of these activity differences was studied (F_{21}). The major line difference was localized to the limbic forebrain and striatum, and it was concluded that decreased cholinergic transmission in these regions may promote motor impairment (Eriksson and Guerri 1986).

Cyclic Nucleotide and Phosphorylation Systems

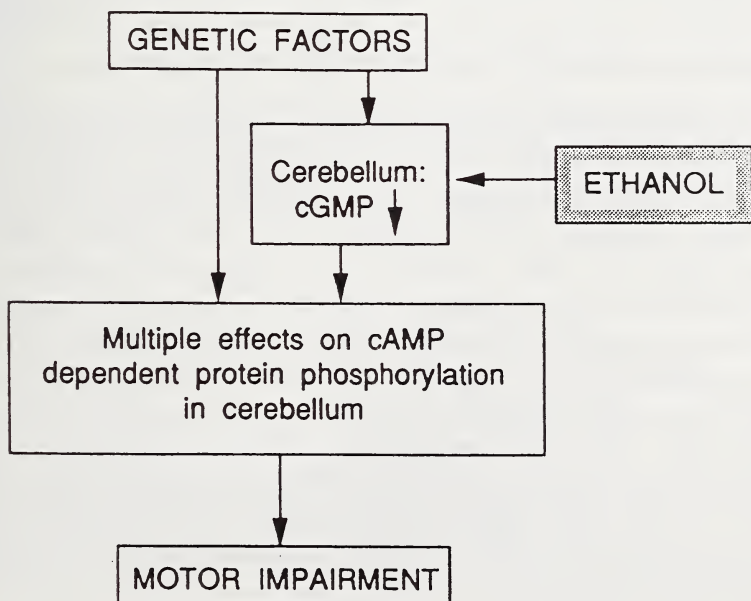
Phosphorylation is known to be involved in a variety of biochemical pathways, hormonal actions, and neuronal transmissions (Greengard 1976; Cohen 1982; Krebs 1985). Characterization of neuronal phosphorylation and its possible role in the mechanism of motor impairment differences between the AT and ANT rat lines was started in generations F_{20} and F_{21} , with the determination of adenylate cyclase activities (Eriksson and Guerri 1986). In this investigation, higher enzyme activities (as measured by cAMP formation by a protein kinase binding assay) were specifically observed in cerebella of the AT rats. Moreover, acute ethanol treatment seemed to inhibit adenylate cyclase activity in the different brain regions, and it was suggested that cerebellar phosphorylation may be a regulator of motor impairment.

The early results with regard to line differences and ethanol effects in cerebellar adenylate cyclase activity were contradicted in later studies in which differences and defects were not detected in enzyme activity (cAMP measured directly by radioimmunology) and in cAMP levels determined after focused microwave irradiation (Eriksson and Allen 1988). In these studies significant line differences were, however, observed in cerebellar cGMP concentrations, with lower levels appear in the ANT rats than in the AT rats. Moreover, acute ethanol treatment was observed to lower drastically the cGMP levels in the different brain regions. Subsequently, it was observed that corresponding cGMP fluctuations could affect the measurements of cAMP-dependent protein kinase activity. In fact, the magnitude of this interference was of such an order that the earlier reported differences and ethanol effects regarding the adenylate cyclase activities, as measured by

kinase binding assays, should be reevaluated. On the basis of the cGMP line differences and the ethanol-induced reductions in cGMP level, this system was suggested to be involved in the mechanisms of ethanol-induced motor impairment.

In addition to merely investigating cyclic nucleotides, we also started screening endogenous phosphorylation in cerebellar preparations from the AT and ANT rats (C.J.P. Eriksson and T.M.K. Allen in preparation). Ethanol treatment (in vivo) caused multiple effects on cAMP-dependent and -nondependent protein phosphorylation. In general, ethanol depressed protein phosphorylation, but also a stimulation of some specific bands was observed. Several line differences appeared, all of which occurred with cAMP-dependent phosphorylation. In general, ethanol depressed protein phosphorylation in the ANT rats more than in the ATs. It is suggested (figure 5) that the mechanism of ethanol-induced motor impairment may involve cGMP-mediated disturbances in cAMP-dependent phosphorylation.

Figure 5.—Possible relationships between cyclic nucleotides, phosphorylation, and motor impairment



Other Aspects

In addition to the aspects described above, some other parameters, such as 5'-nucleotidase, (Na+K)-ATPase, sodium channels, glucose uptake, hippocampal electroencephalogram and serum corticosterone, have been determined. 5'-Nucleotidase and (Na+K)-ATPase activities were determined from the same F₂₀ animals as were the other membrane enzymes, acetylcholinesterase and adenylate cyclase (Eriksson and Guerri 1986). Slightly higher 5'-nucleotidase activities were observed in the cerebella of the ANT rats than of the ATs, and it was concluded that adenosine mechanisms may be involved in ethanol-induced motor impairment.

According to the (Na+K)-ATPase results from F₂₀, a sex-dependent line difference appeared, with the AT males displaying more enzyme activity than the ANT males; therefore, involvement of (Na+K)-ATPase in the mechanism of motor impairment is possible (Eriksson and Guerri 1986). Another aspect of the sodium flux has more recently (F₂₈) been compared in the AT and ANT rats (Korpi et al. 1988). In this study, no line differences in the binding to the neurotoxin receptor site 2 were observed. It was concluded that the genetic selection has not involved the genes or expression of the genes associated with the voltage-sensitive sodium channels.

To test neuronal glucose utilization in the AT and ANT rat lines, the 2-deoxy-D-glucose technique was applied in generations F₂₅₋₂₆ (Korpi et al. 1987a,b; Lindroos and Korpi 1988). No line differences were observed in the first study with regard to basal utilization and to the lowering effect of ethanol (Korpi et al. 1987). However, in the subsequent investigation, in which the different brain regions were dissected in more detail, some line differences appeared (Lindroos and Korpi 1988). Especially in the ANT rats, areas associated with sensory input were damped but motor relay nuclei were relatively active, which suggested a tendency to motor overactivity relative to sensory input. The ANT rats furthermore showed slightly less relative damping of cortical associative areas and differences in limbic structures than did the ATs, and it was suggested that the higher level of ethanol-induced motor impairment of the ANT rats may relate to defects in their integration of sensory and motor processes.

In the electrophysiologic study of the AT and ANT rat lines (F_{26}), hippocampal rhythmic slow activity (RSA) was recorded during rotation and vibration stimulation after treatment with saline and ethanol (Kaheinen et al. 1988). The saline-treated ANT rats had more high-frequency RSA than did the ATs, especially during the rotational stimulation. The differences were not found during the ethanol sessions. Plasma corticosterone levels were higher in the ATs than in the ANTs after the recording sessions. The possible role of these findings was left open for future investigations.

Summary

Several behavioral and neurochemical differences have been reported in investigations of our rat lines selectively outbred for low (AT) and high (ANT) degree of ethanol-induced motor impairment. This paper has described these differences and their possible relationship to the motor impairment differences. The main differences are schematized in figure 6. The negative correlation between motor impairment and the other behaviors, basal activity and ethanol drinking behavior, may be true but must be verified in further detail.

Possible associations between motor impairment and increased dopaminergic activity, decreased cholinergic activity, disturbed GABAergic activity, reduced cGMP, and disturbed phosphorylation have been reported. The relationship between these systems and the mechanisms by which they affect genetic differences in sensitivity will be challenging topics for future studies.

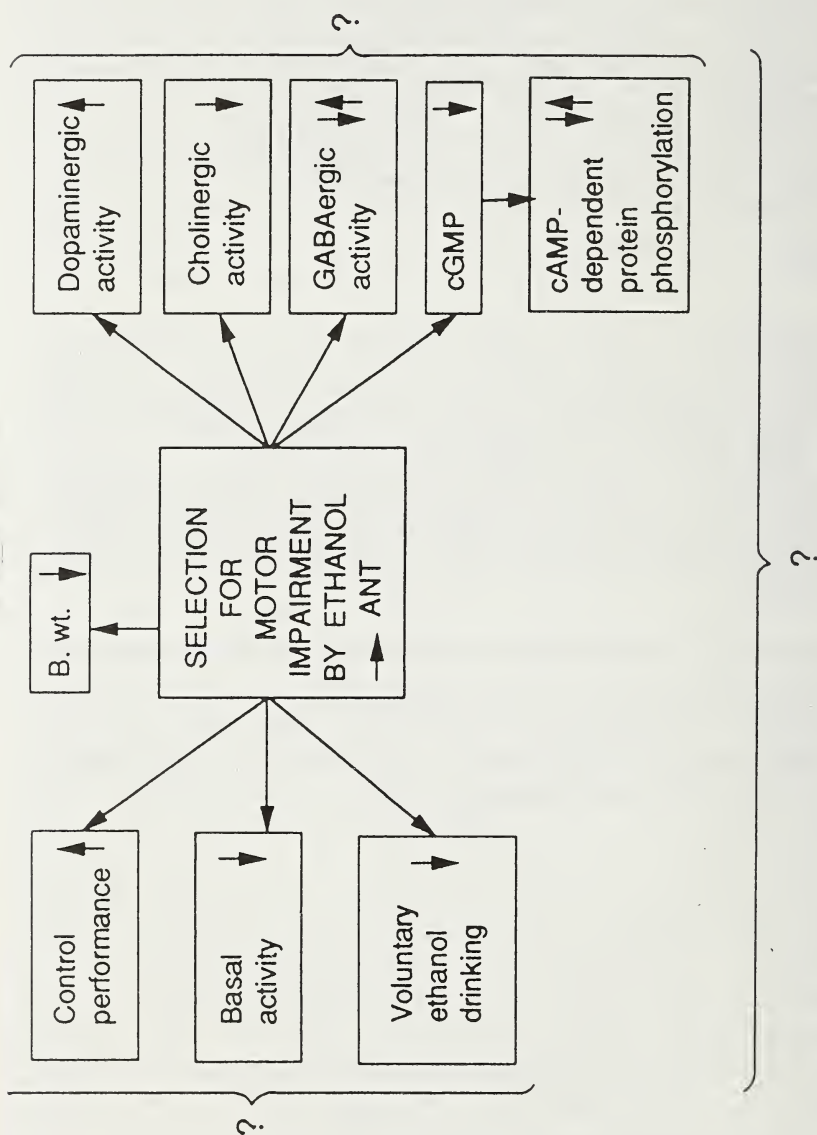


Figure 6.—Possible relationships between motor impairment, other behaviors, and neurochemical factors

References

- Ahtee, L.; Attila, L.M.J.; and Kiianmaa, K. Brain catecholamines in rats selected for their alcohol behavior. In: Eriksson, K.; Sinclair, J.D.; and Kiianmaa, K., eds. *Animal Models in Alcohol Research*. New York: Academic Press, 1980. pp. 51-55.
- Bass, M.B., and Lester, D. Tolerance to ethanol-induced impairment of water escape in rats bred for ethanol sensitivity. *Psychopharmacology* (Berlin) 71:153-158, 1980.
- Belknap, J.K. Genetic factors in the effects of alcohol: Neurosensitivity, functional tolerance and physical dependence. In: Riger, H., and Crabbe, J.C., eds. *Alcohol Tolerance and Dependence*. New York: Elsevier, 1980, pp. 157-180.
- Bonetti, E.P.; Pieri, L.; Cumin, R.; Schaffner, R.; Pieri, M.; Gamzu, E.K.; Muller, R.K.M.; and Haefely, W. Benzodiazepine antagonist Ro 15-1788: Neurological and behavioral effects. *Psychopharmacology* (Berlin) 78:8-18, 1982.
- Cohen, P. The role of protein phosphorylation in neuronal and hormonal control of cellular activity. *Nature* (London) 296:613-620, 1982.
- Crabbe, J.C. Sensitivity to ethanol in inbred mice: Genotypic correlations among several behavioral responses. *Behav Neurosci* 97:280-289, 1983.
- Crabbe, J.C.; Janowsky, J.S.; Young, E.R.; Kosobud, A.; Stack, J.; and Riger, H. Tolerance to ethanol hypothermia in inbred mice: Genotypic correlations with behavioral responses. *Alcoholism* 6:446-458, 1982.
- Crabbe, J.C.; Kosobud, A.; Tam, B.R.; Young, E.R.; and Deutsch C.M. Genetic selection of mouse lines sensitive (cold) and resistant (hot) to acute ethanol hypothermia. *Alcohol Drug Res* 7:163-174, 1987a.
- Crabbe, J.C.; Young, E.R.; Deutsch, C.M.; Tam, B.R.; and Kosobud, A. Mice genetically selected for differences in open-field activity after ethanol. *Pharmacol Biochem Behav* 27:577-581, 1987b.

- Eriksson, C.J.P. Finnish selection studies on alcohol-related behaviors: Factors regulating voluntary alcohol consumption. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. ADM(81-1113). Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 119-145.
- Eriksson, C.J.P., and Allen, T.M.K. Phosphorylation and genetically-determined variation in ethanol intoxication. *Alcohol Alcoholism* 23:A7, 1988.
- Eriksson, C.J.P.; Deitrich, R.A.; Rusi, M.; Clay, K.; and Petersen, D.A. In: Yoshida, H.; Hagihara, Y.; and Ebashi, S., eds. *Advances in Pharmacology and Therapeutics II*. Vol 5. *Toxicology and Experimental Models*. New York: Pergamon Press, 1982. pp. 245-251.
- Eriksson, C.J.P., and Guerri, C. Neuronal membrane enzymes in rat lines selected for differential motor impairment by ethanol. *Pharmacol Biochem Behav* 24:1115-1121, 1986.
- Eriksson, K., and Rusi, M. Finnish selection studies on alcohol-related behaviors: General outline. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. ADM(81-1113). Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 87-117.
- Eriksson, C.J.P., and Sarviharju, M. Motor impairment, narcosis and hypothermia by ethanol: Separate genetic mechanisms. *Alcohol* 1:59-62, 1984. Goedde, H.W.; Agarwal, D.P.; and Harada, S. Pharmacogenetics of alcohol sensitivity. *Pharmacol Biochem Behav* 18(Suppl 1):161-166, 1983.
- Goedde, H.W.; Agarwal, D.P.; and Harada, S. Pharmacogenetics of alcohol sensitivity. *Pharmacol Biochem Behav* 18 (Suppl 1):161-166, 1983.
- Greengard, P. Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters. *Nature* (London) 260:101-108, 1976.

- Hellevuo, K., and Kiianmaa, K. Effects of ethanol, barbital and lorazepam on brain monoamines in rat lines selectively outbred for differential sensitivity to ethanol. *Pharmacol Biochem Behav* 29:183-188, 1988.
- Hellevuo, K.; Kiianmaa, K.; Juhakoski, A.; and Kim, C. Intoxicating effects of lorazepam and barbital in rat lines selected for differential sensitivity to ethanol. *Psychopharmacology* (Berlin) 91:263-267, 1987.
- Hellevuo, K., and Korpi, E.R. Failure of Ro 15-4513 to antagonize ethanol in rat lines selected for differential sensitivity to ethanol and in Wistar rats. *Pharmacol Biochem Behav* 30:183-188, 1988.
- Hunt, W.A., and Majchrowicz, E. Alterations in neurotransmitter function after acute and chronic treatment with ethanol. In: Majchrowicz, E., and Noble, E.P., eds. *Biochemistry and Pharmacology of Ethanol*. Vol. 2. New York: Plenum Press, 1979. pp. 167-185.
- Kaheinen, P.; Korpi, E.R.; Pyykkö, I.; Mäntysalo, S.; and Ignatius, J. Hippocampal rhythmic slow activity in rat lines selected for differences in ethanol-induced motor impairment. *Pharmacol Biochem Behav* 30:177-181, 1988.
- Kiianmaa, K. Brain monoamine functions in rat lines selected for differences in alcohol-related behaviors. *Acta Pharmacol Toxicol* 55(Suppl. 1):14, 1984.
- Kiianmaa, K.; Hellevuo, K.; and Korpi, E.R. The AT (alcohol tolerant) and ANT (alcohol nontolerant) rat lines selected for differences in sensitivity to ethanol: An overview. In: Kuriyama, K.; Takada, A.; and Ishii, H., eds. *Biomedical and Social Aspects of Alcohol and Alcoholism*. New York: Elsevier, 1988. pp. 415-418.
- Kiianmaa, K., and Tabakoff, B. Catecholaminergic correlates of genetic differences in ethanol sensitivity. In: Usdin, E., Carlsson, A., Dahlström, A., and Engel, J., eds. *Catecholamines: Neuropharmacology and Central Nervous System Theoretical Aspects*. New York: Alan R. Liss, 1984. pp. 145-151.

Selective Breeding for Initial Sensitivity

- Korpi, E.R.; Lindroos, F.; Pyykkö, I.; Malminen, O.; Kaheinen, P.; Ignatius, J.; and Mäntysalo, S. Ethanol-induced modulation of hippocampal EEG and cerebral 2-deoxyglucose accumulation in rat lines with differing alcohol sensitivities. *Alcohol Alcoholism Suppl* 1:713-717, 1987a.
- Korpi, E.R.; Reith, M.E.A.; and Lajtha, A. Synaptosomal sodium channels in rat lines selected for alcohol-related behaviors. *Alcohol* 5:81-84, 1988.
- Korpi, E.R.; Sinclair, J.D.; and Malminen, O. Dopamine D₂ receptor binding in striatal membranes of rat lines selected for differences in alcohol-related behaviours. *Pharmacol Toxicol* 61:94-97, 1987b.
- Krebs, E.G. The phosphorylation of proteins: A major mechanism for biological regulation. *Biochem Soc Trans* 13:813-820, 1985.
- Le, A.D., and Kiianmaa, K. Initial sensitivity and the development of acute and rapid tolerance to ethanol in the AT and ANT rats. In: Kiianmaa, K.; Tabakoff, B.; and Saito, T., eds. *Genetic Aspects of Alcoholism, The Finnish Foundation for Alcohol Studies*. Vol. 37. Helsinki: Painokaari Oy, 1989. pp. 147-155.
- Li, T.-K.; Lumeng, L.; McBride, W.J.; and Waller, M.B. Progress toward a voluntary oral consumption model of alcoholism. *Drug Alcohol Depend* 4:45-60, 1979.
- Li, T.-K.; Lumeng, L.; McBride, W.J.; and Waller, M.B. Indiana selection studies on alcohol-related behaviors. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. ADM(81-1113). Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 171-191.
- Lindroos, F., and Korpi, E.R. Effects of moderate ethanol sedation on brain regional 2-deoxyglucose uptake in alcohol- sensitive and alcohol-insensitive rat lines. *Pharmacol Biochem Behav* 30:781-786, 1988.
- Malila, A. Intoxicating effects of three aliphatic alcohols and barbital on two rat strains genetically selected for their ethanol intake. *Pharmacol Biochem Behav* 8:197-201, 1978.

- Malminen, O., and Korpi, E.R. GABA/benzodiazepine receptor/chloride ionophore complex in brains of rat lines selectively bred for differences in ethanol-induced motor impairment. *Alcohol* 5:239-249, 1988.
- McClearn, G.E., and Anderson, S.M. Genetics and ethanol tolerance. *Drug Alcohol Depend* 4:61-76, 1979.
- McIntyre, T.D., and Alpern, H.P. Reinterpretation of the literature indicates differential sensitivities of long-sleep and short-sleep mice are not specific to alcohol. *Psychopharmacology* (Berlin) 87:379-389, 1985.
- Middaugh, L.D.; Read, E.; and Boggan, W.O. Effects of naloxone on ethanol-induced alterations of locomotor activity in C57BL/6 mice. *Pharmacol Biochem Behav* 9:157-160, 1978.
- National Institute on Alcohol Abuse and Alcoholism. *Development of Animal Models as Pharmacogenetic Tools*, McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. DHHS Pub. No. (ADM)81-1133. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981.
- Nikander, P., and Pekkanen, L. An inborn alcohol tolerance in alcohol-preferring rats. The lack of relationship between tolerance to ethanol and the brain microsomal (Na⁺K⁺)ATPase activity. *Psychopharmacology* (Berlin) 51:219-223, 1977.
- Pispa, J.P.; Huttunen, M.O.; Sarviharju, M.; and Ylikahri, R. Enzymes of catecholamine metabolism in the brains of rat strains differing in their preference for or tolerance of ethanol. *Alcohol Alcoholism* 21:181-184, 1986.
- Pohorecky, L.A., and Brick, J. Pharmacology of ethanol. *Pharmacol Ther* 36:335-427, 1988.
- Propping, P. Genetic control of ethanol action on the central nervous system. *Hum Genet* 35:309-334, 1977a.
- Propping, P. Psychophysiologic test performance in normal twins and in a pair of identical twins with essential tremor that is suppressed by alcohol. *Hum Genet* 36:321-325, 1977b.

- Riley, E.P.; Worsham, E.D.; Lester, D.; and Freed, E.X. Selective breeding of rats for differences in reactivity to alcohol. An approach to an animal model of alcoholism. II. Behavioral measures. *J Studies Alcohol* 38:1705-1717, 1977.
- Rusi, M.; Eriksson, K.; and Mäki, J. Genetic differences in the susceptibility to acute ethanol intoxication in selected rat lines. In: Gross, M.M., ed. *Alcohol Intoxication and Withdrawal*. Vol. IIIA. New York: Plenum Press, 1977. pp. 97-109.
- Sinclair, J.D.; Rusi, M.; Airaksinen, M.M.; and Altshuler, H.L. Relating TIQ's, opiates, and ethanol. In: Bloom, F.E.; Barchas, J.; Sandler, M.; and Usdin, E., eds. *Beta-Carbolines and Tetrahydroisoquinolines*. New York: Alan R. Liss, 1982. pp. 365-376.
- Sinclair, J.D.; Viitamaa, T.; and Hyytiä, P. Behavioral and color variations between rat lines developed for differential alcohol sensitivity. *Alcohol Alcoholism Suppl* 1:449-453, 1987.
- Tabakoff, B., and Hoffman, P.L. Alcohol and neurotransmitters. In: Rigter, H., and Crabbe, J.C., eds. *Alcohol Tolerance and Dependence*. New York; Elsevier, 1980. pp. 201-226.
- Tabakoff, B.; Ritzmann, R.F.; Raju, T.S.; and Deitrich, R.A. Characterization of acute and chronic tolerance in mice selected for inherent differences in sensitivity to ethanol. *Alcoholism* 4:70-73, 1980.
- Ticku, M.K., and Kulkarni, S.K. Molecular interactions of ethanol with GABAergic system and potential of Ro 15-4513 as an ethanol antagonist. *Pharmacol Biochem Behav* 30:501-510, 1988.
- Wallgren, H., and Barry, H., III. Voluntary selection of alcohol. In: Wallgren, H., and Barry, H., eds. *Actions of Alcohol*. Vol. 2. *Chronic and Clinical Aspects*. New York: Elsevier, 1970. pp. 403-477.

Selective Breeding of Rats Differing in Sensitivity to the Hypnotic Effects of Acute Ethanol Administration¹

*Karen Spuhler, Richard A. Deitrich,
and Rodney C. Baker*²

Introduction

Selective breeding has provided a powerful approach for the genetic analysis of specific primary phenotypes and correlated secondary traits, especially in pharmacogenetics and the study of alcoholism. The procedure uses repeated selection pressure, ideally over a period of at least 20 generations. As selection proceeds, genes at loci controlling the primary selected genotypes become fixed, whereas genes at nonassociated loci approach homozygosity at a slower rate as a result of genetic drift (finite population size). One drawback in the use of selective breeding in laboratory rodents has been the frequent lack of a complete design that includes replicate lines in each direction of response and concurrent replicate unselected control lines. If feasible, a selection study should use both replicate lines (to strengthen

¹This research was supported by funds from U.S. Public Health Service grants AA05868, AA03527, and AA07157.

²Department of Pharmacology, University of Colorado School of Medicine, Denver, CO 80262.

conclusions about the response to selection and correlated traits) and control lines (to measure phenotypic changes related to environmental effects) (DeFries 1981).

In addition, a systematically produced genetically heterogeneous stock (HS) has not always been used as the base population to initiate selection. An HS line of rats is now available from the Animal Resource Center at the National Institutes of Health (NIH), Bethesda, MD; it was developed from the intercrossing of eight inbred strains (ACI, BN, BUF, F344, M520, MR, WKY, and WN; Hansen and Spuhler 1984). The HS (N/NIH) rats and their progenitor strains have exhibited genetic variation for alcohol-related phenotypes (Spuhler and Deitrich 1984), including alcohol preference (Li and Lumeng 1984), initial sensitivity to acute doses of ethanol (Deitrich and Baker 1984; Palmer et al. 1987), ethanol metabolism (Erickson 1984; Palmer et al. 1987), development of tolerance (Tabakoff and Culp 1984), and sensitivity of the cerebellar Purkinje neuron (PN) to local application of ethanol *in situ* (Palmer et al. 1987). Moreover, a significant genetic correlation between initial sensitivity and cerebellar PN sensitivity was found (Palmer et al. 1987).

The selective breeding of rats for initial sensitivity to acute alcohol was initiated in order to produce a larger and more convenient animal model for studying the actions of alcohol in the central nervous system (CNS) and to determine the commonality of ethanol response in an additional species relative to the responses of the LS and SS mice. With the focus on neurologic experiments, we have established an ongoing selective breeding program. As progress continues toward a divergence of response for sensitivity to acute doses of ethanol, a collaborative effort with other investigators has ensured the periodic monitoring of secondary alcohol-related phenotypes since the beginning of the study. These additional studies have provided important findings on changes in the response of correlated traits over the generations of breeding.

Methods

Animals from the HS rat population at NIH were used as the base population from which to begin selective breeding. The animals are housed

in standard rat cages in assigned cubicles within a larger colony room. Weaning is completed between 23 and 26 days of age, and testing of animals is started at 60 days of age. A 12-hr light/dark cycle is used, and the humidity of the colony room is routinely checked. Testing of animals for behavioral sensitivity to alcohol is done during the daylight hours.

Testing of Initial Sensitivity to Acute Doses of Ethanol

The protocol for estimating the hypnotic response to an anesthetic dose of ethanol is similar to that used in the selective breeding of the LS and SS mice (McClearn and Kakihana 1981). A maximum of 25 animals are tested in 1 session, which includes 2-3 litters, each from separate lines; 2-3 sessions are completed in 1 day. In each generation, four to five males and females from each litter of each line are tested for initial sensitivity. An ethanol dose of 3.5 g/kg (20 percent [wt/vol] in saline) was used in testing the HS animals for the base population. In generations 1-8 of selection, an ethanol dose of 3.0 g/kg (15 percent [wt/vol] in saline) was used. Immediately after recovery of the righting reflex (RR), each animal was sampled for blood ethanol concentration (BEC). These measurements were done by an enzymatic method (Bonnichsen and Brink 1955).

Selective Breeding Protocol

The base population consisted of 44 individual litters from the HS to construct a within-litter selection design. Males and females from these litters were tested for sleep time and formed the population from which the lines were initiated. Six separate lines were established, including two replicate low-sensitivity lines (LAS-1 and -2), two replicate high-sensitivity lines (HAS-1 and -2), and two replicate control lines (CAS-1 and -2). To maintain 10 mating pairs per line as selection progresses, 14-16 pairs were set up originally. Sixteen litters were used to begin the LAS-1 and HAS-1 lines. The male and female from each litter with the shortest duration of sleep time were selected to be parents for LAS-1; likewise, the male and female from each litter with the longest duration of sleep time were selected as parents for HAS-1. By using the same approach, males and females from another set of 16 litters were selected as parents for LAS-2 and HAS-2. Parents from CAS-1 were chosen without regard to ethanol sensitivity from among 14 of the 32

litters used to establish the high and low lines; 1 male and 1 female were selected at random from an additional set of 15 litters as parents for CAS-2. A rotating mating paradigm, which minimizes common ancestry as much as possible, is used. Eight generations of selective breeding have been completed; there remain 13 litters in LAS-1, 11 in LAS-2, 10 in HAS-1, 12 in HAS-2, 9 in CAS-1, and 12 in CAS-2.

Experimental Procedures for Ethanol-Related Phenotypes

ELECTROPHYSIOLOGIC RECORDING OF CEREBELLAR PN SENSITIVITY (PN SENS)

The in situ experimental procedure is similar to that previously used in the mouse studies and has been described in detail elsewhere (Spuhler et al. 1982, Palmer et al. 1987). The sensitivity is measured as the ethanol dose (pounds per square inch x seconds), required to elicit an average 50 percent depression of PN firing rate. Five animals per line and six neurons per animal were tested.

ETHANOL METABOLISM (METAB)

Animals were administered ethanol (3.0 g/kg) intraperitoneally (IP), and retro-orbital blood samples were taken at 0.5, 1, 2, 3, and 4 hr after the ethanol dose. Metabolism was estimated as the mean decrease in BEC with time and calculated by the Widmark method (Watson 1989). Twelve animals from LAS-1 and -2, 11 from HAS-1, 10 from HAS-2, 9 from CAS-1, and 11 from CAS-2 were tested.

BEC AT LOSS OF THE RIGHTING REFLEX (BEC AT LRR)

Animals were administered 10 g/kg of ethanol intragastrically with a 30 percent (wt/vol) solution of ethanol in water. The time to LRR was monitored; when loss occurred, 40- μ l retro-orbital blood samples were taken for BEC analysis. Eight animals per line were tested.

FIFTY PERCENT EFFECTIVE DOSE FOR LRR (ED_{50} LRR)

Animals were administered a single dose of ethanol IP (2.25, 2.50, 2.625, or 2.75 g/kg; 20 percent [wt/vol] in saline). At 15 min postinjection, animals were scored as positive or negative for LRR, and 40- μ l retro-orbital blood samples were taken to determine the BEC. Fifty animals per line were tested. The percentage of animals with LRR and their respective average BEC values were used to estimate the ED_{50} (Litchfield and Wilcoxon 1949).

SENSITIVITY TO ACUTE ADMINISTRATION OF PENTOBARBITAL

Animals were given a single IP dose of sodium pentobarbital (60 mg/kg) in saline. The sleep time interval was determined, and at recovery of the righting reflex, a 40- μ l retro-orbital blood sample was taken for assay of blood pentobarbital levels. There were 12 animals from LAS-1 and -2, 12 from HAS-1 and -2, and 6 from CAS-1 and -2 tested. Butabarbital (10 μ g) was added to each sample as an internal standard. Samples were diluted, acidified with HCl, and extracted with methylene chloride. The organic phase was back-extracted with 0.1 N NaOH. The aqueous phase was acidified and reextracted with methylene chloride. Samples were taken to dryness, derivatized by using methyl iodide-potassium carbonate in acetone (Knapp 1979), and analyzed by gas chromatography.

ADRENAL CORTICOSTERONE RESPONSE TO ACUTE ETHANOL ADMINISTRATION

Animals were handled and sham injected IP on a daily bases for 7 days. On day 8 they were injected either with saline or with 3.0 g/kg (15 percent [wt/vol] in saline) of ethanol. Blood samples were taken 30 min later upon decapitation and analyzed for corticosterone levels. Three animals from each replicate line were tested with either saline or ethanol. Plasma corticosterone was extracted with ethanol and quantified by radioimmunoassay (Zgombick and Erwin 1987).

Results and Discussion

The mean sleep time of the HS males from which parents were chosen to initiate the lines was 161 ± 6 min; the mean sleep time of the HS females was 149 ± 5 min. These sensitivity responses correspond to a dose of 3.5 g/kg and are somewhat higher than those reported by Deitrich and Baker (1984). Because of the extensive sleep time interval represented in the base population, the ethanol dose for generation 1 and thereafter was lessened to 3.0 g/kg.

Figure 1 depicts the response to selection for sleep time in the replicate lines of the LAS and HAS animals, and the average of the two replicate CAS lines through eight generations of selection at an ethanol dose of 3.0 g/kg. The generation means of the lines indicate a consistent, progressive divergence of the high- and low-sensitivity lines. The replicates also appear fairly homogeneous in each direction. As the HAS and LAS lines separate over the generations, the CAS lines have fluctuated in an intermediate range, which is expected of an unselected, genetically heterogenous population for a trait under polygenic control.

By regressing the sleep time means of each selected line on the cumulative selection differential and the mean of the control lines for each generation (Spuhler et al. 1987; Hill 1972), an estimate of the realized heritability is computed. The estimated values were as follows: LAS-1, 0.14; LAS-2, 0.21; HAS-1, 0.36; and HAS-2, 0.33. The overall realized heritability was 0.26, which is consistent with estimates of additive genetic variation from other alcohol-related selection studies.

The frequency distribution of sleep time for generation 8 in the pooled replicates of the LAS, HAS, and CAS lines is shown in figure 2. The range of response is large, extending from 10 to 170 min. A gradual separation of the LAS and HAS lines is evident, although a considerable amount of overlap is apparent. The degree of overlap between the two distributions is expected to decrease as selective breeding continues. The CAS lines are showing a response over a large portion of the total range, with skewness toward the shorter duration. As a "floor and ceiling effect" develops with further selection, the doses of ethanol will need to be increased for the LAS rats and decreased for the HAS rats; however, this change in ethanol administration

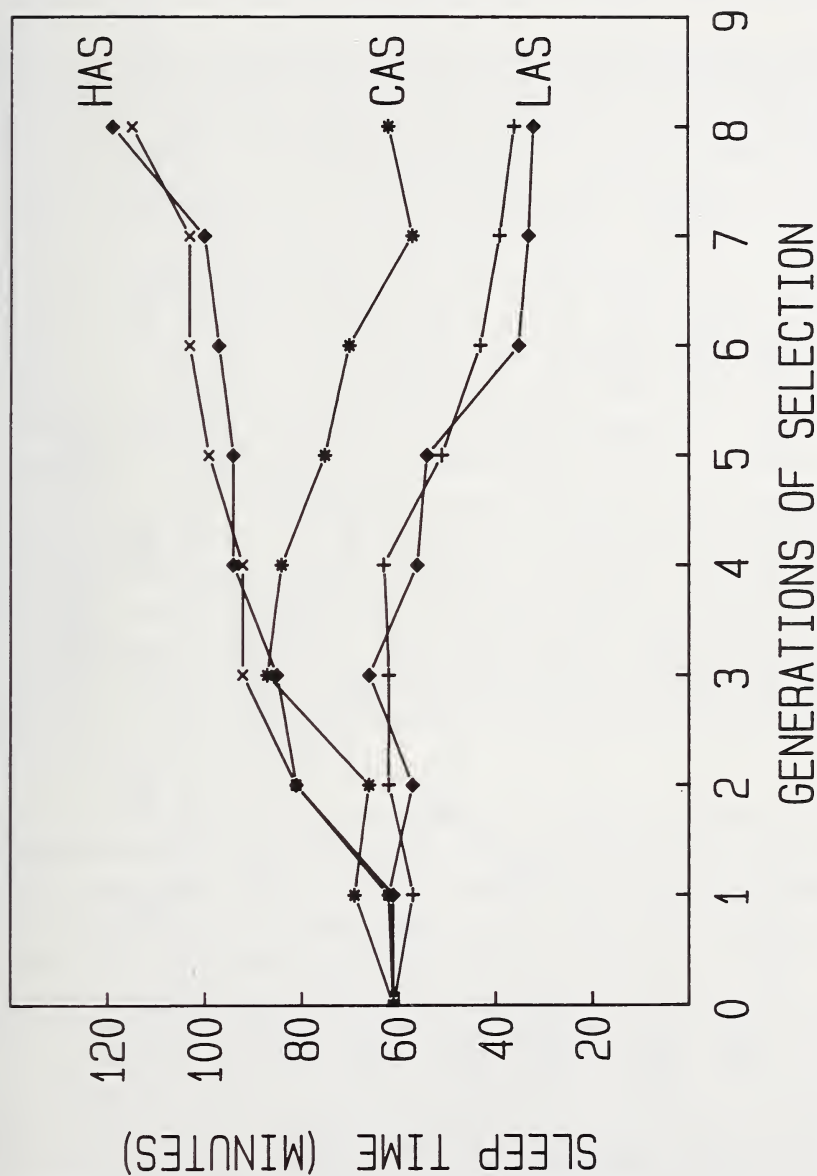


Figure 1.—Response to selection after eight generations in LAS, CAS, and HAS rat lines.

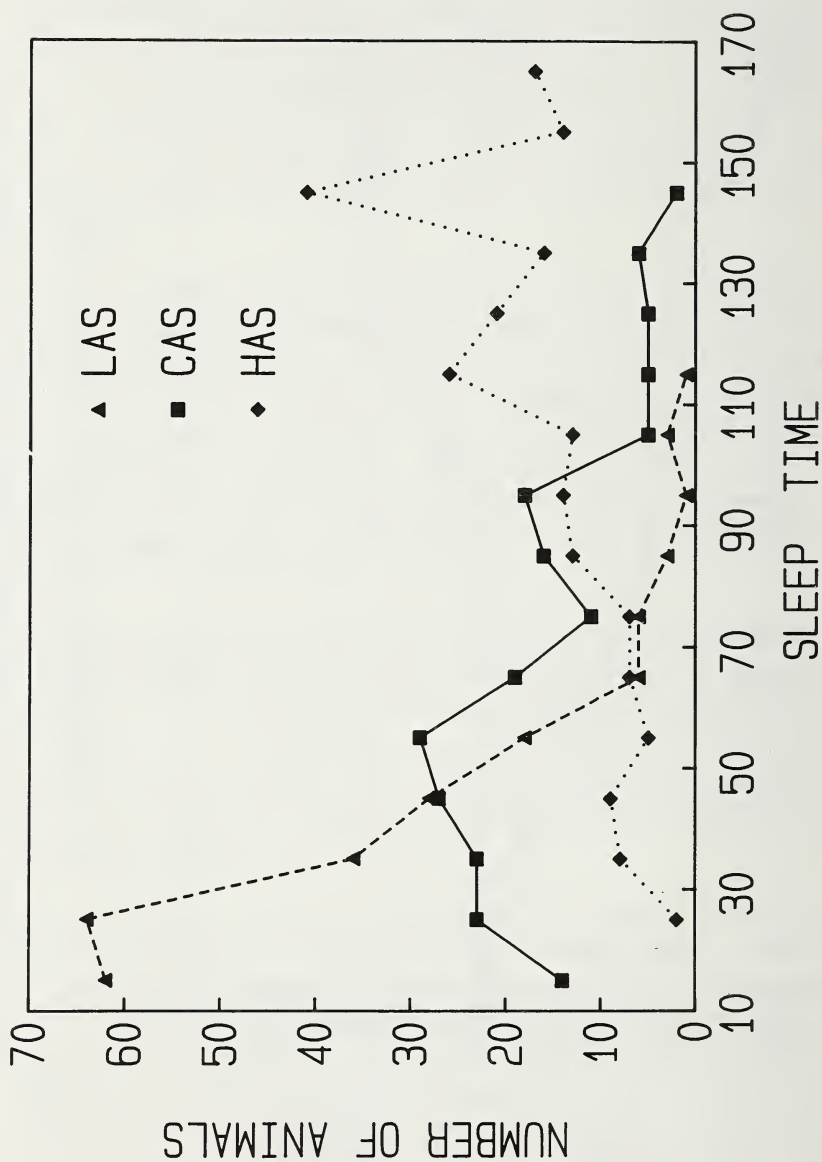


Figure 2.—Sleep time distribution of generation 8 animals from the LAS, CAS, and HAS selected lines.

will be delayed as long as is reasonable for measurement of an accurate sleep time response.

The mean responses of the selected and control lines at generations 7 and 8 for the various secondary alcohol-related phenotypes, as well as sleep time, are listed in table 1. At generation 8, the mean sleep time for both LAS lines is 34 min, whereas that for both HAS lines is 117 min, almost 3.5 times longer. The two CAS lines are intermediate at 62 min. The BEC at recovery of the righting reflex (BEC at RR) also exhibits an average difference between the lines such that the LAS level is higher at 87 mM and the HAS level is lower at 75 mM; the CAS level is 82 mM. These findings support those previously reported in that animals with shorter sleep times recover at higher BEC than do those with longer sleep times, and they strengthen the relative importance of the CNS in the hypnotic response to alcohol. At generation 8, the ED_{50} LRR also differed between the LAS and HAS lines; the BEC at 15 min, corresponding to a loss in 50 percent of the animals, was an average of 77 mM in the two LAS lines and 52 mM in the two HAS lines. The CAS lines were similar to the HAS lines, with a BEC of 56 mM. Thus, the HAS animals lose the reflex at a lower BEC than do the LAS animals, supporting the divergence in sleep time that the lines are exhibiting. Similarly, the two LAS lines had a higher BEC at LRR (74 mM) than did the two HAS lines (64 mM), with the value for the two CAS lines very close (66 mM) to that of the HAS lines.

The HAS and LAS lines were also found to differ significantly in the depressant effects of pressure-ejected ethanol on PN Sens at generation 8 in that a mean dose of 204 psi x s was required to elicit a 50 percent inhibition of the firing rate of PN in the LAS rats and a dose of only 43 psi x s was required in the HAS rats. The CAS animals were intermediate (81 psi x s) to the high and low lines but somewhat closer to the more sensitive end. The correlation of individual animal sleep time and PN Sens, pooled over lines, was -0.79 ± 0.13 . The differential response in the cerebellar PN to ethanol depression also had been observed at generation 3 (Palmer et al. 1987). These data strongly support the evidence for a genetic correlation between this CNS sensitivity index and sleep time demonstrated in both the rat and mouse inbred strains (Spuhler et al. 1982; Palmer et al. 1987).

Table 1.—Phenotypic responses of alcohol-sensitive and -insensitive selected lines of rats in generations 7 and 8

Phenotype	LAS-1	LAS-2	HAS-1	HAS-2	CAS-1	CAS-2
Ethanol sleep time	36±3	32±4	19±6	115±8	68±6	55±7
BEC at RR	87±1	87±1	73±2	76±1	81±1	83±1
ED ₅₀ LRR	77±8		52±6		56±6	
BEC at LRR	73±4	75±2	63±4	64±3	64±3	67±4
PN Sens	201±39	206±21	47±12	39±9	81±18	
Metab	14.2±1	12.5±1	13.0±1	13.1±1	12.9±1	13.2±1
Corticosterone saline	146±26		291±52		266±37	
Ethanol	366±24		381±51		323±24	
Barbiturate sleep time	129±9		182±11		156±19	
Blood levels	20.8±1		16.4±1		18.2±1	

With regard to Metab, the HAS, CAS, and LAS replicate lines were found to have similar clearance rates at generation 7. This was also found to be the case in an earlier small sampling at generation 2 (LAS, 16.3 mmol/hr; HAS, 13.0 mmol/hr; and CAS, 13.0 mmol/hr). Although genetic variation for

Metab was indicated in the previous study of the inbred strains (Palmer et al. 1987), selection pressure has not yet resulted in a notable, consistent difference between the high and low lines for this secondary phenotype. A similar trend was found for the LS and SS mice during their selective breeding (Smolen et al., 1986; Erwin et al. 1976). This finding does not preclude the fact that genetic segregation for Metab could occur during the later generations of selection, in addition to continued divergence of both behavioral and neurologic sensitivity to ethanol. Moreover, these responses could be symmetric or asymmetric relative to the progression of changes in phenotype compared with the control response. It is reasonable to expect that in future HAS and LAS rats, large genetic differences will be observed for behavioral sedation, CNS depressant effects, and liver ethanol clearance rate.

At generation 7, both HAS and CAS lines had increased corticosterone levels (in nanograms per milliliter) after a saline injection, whereas all lines had a quite large increase in levels after an ethanol injection. Marginal differences between the lines were not as clear cut as for the secondary phenotypes at this point in the study.

The lines showed differences in pentobarbital sleep time in the predicted manner of HAS>CAS>LAS with the reverse ranking for blood pentobarbital levels (in micrograms per milliliter) (table 1). Interestingly, this sleep time ranking for the rat lines is dissimilar to that found for the LS and SS mice in previous studies (Howerton et al. 1984; Dudek et al. 1984; Alpern and McIntyre 1986) in that the SS mice exhibited either greater or the same sleep time compared with LS mice at a 60-mg/kg dose. The basis for this contrast in barbiturate sensitivity is unclear at present.

Table 2 shows a comparison of the HAS and LAS rat lines with the LS and SS mouse lines. In additional studies, the LAS and HAS rats have been found to differ in (1) cerebellar norepinephrine levels (French et al. 1988a), similar to values found for the mouse lines (French et al. 1988b; Spuhler et al. 1987; Erwin and Cornell 1986), and (2) the increase in adrenal tyrosine hydroxylase activity after acute ethanol administration (French et al. 1988a), similar to findings for the mouse lines (French et al. 1988b).

Table 2.—Comparison of HAS and LAS rat selected lines with SS and LS mouse lines

Phenotype	Rat	Mouse
Sleep time		
Ethanol	LAS < HAS	SS < LS
Pentobarbital	LAS < HAS	SS > LS
Metab	LAS = HAS	SS = LS
PN Sens	LAS < HAS	SS < LS
Cerebellar norepinephrine levels	LAS > HAS	SS > LS
Ethanol—adrenal tyrosine hydroxylase	LAS < HAS	SS < LS
Brain ascorbic acid levels	LAS < HAS	SS = LS
Corticosterone		
Saline	LAS < HAS	SS < LS
Ethanol	LAS = HAS	SS < LS
GABA Cl ⁻ influx		
Muscimol only	LAS = HAS	SS < LS
Ethanol + muscimol	LAS < HAS	SS < LS

A contrasting finding between the mouse and rat lines has been reported (Deitrich et al. 1988) for brain ascorbic acid levels. LAS rats were found to have less ascorbate than HAS rats at generation 5, whereas, no differences between the LS and SS mice were found. These puzzling findings will be explored further in subsequent generations of the rat selected lines.

A most interesting finding in the HAS and LAS lines, substantiating the results from studies of LS and SS mice, is the differences in ethanol potentiation of muscimol-stimulated Cl⁻ influx in both cortical and cerebellar membranes (Allan et al. 1988). Ethanol in concentrations of 10-30 mM potentiated the γ -aminobutyric acid (GABA) agonist-dependent increase in

Cl⁻ influx only in the HAS lines, not in the LAS lines. These results support the putative role of the sensitivity of GABA-operated Cl⁻ channels in the hypnotic effects of ethanol.

Conclusion

Selective breeding of rats for high and low sensitivity to acute administration of ethanol has resulted in a progressive divergence of the replicate HAS and LAS lines through generation 8. The monitoring of secondary ethanol-related phenotypes has demonstrated that as selection for sleep time continues, divergence of these traits occurs as well. These findings, which measure differing aspects of initial sensitivity to alcohol, provide strong support for a genetic correlation of these various phenotypes. These rat selected lines also have simulated the LS and SS mouse lines and provide an additional animal model for ascertaining the mechanism of action of ethanol. The suggested species generality of these overall findings from selective breeding encourages further study of the genetic basis of alcoholism. We urge others engaged in research on the effects of alcohol to pursue experimental designs that use these selected lines in order to compile a growing data base on the interrelationships of the various actions of ethanol.

References

- Allan, A.M.; Spuhler, K.P.; and Harris, R.A. Gamma-aminobutyric acid-activated chloride channels: Relationship to genetic differences in ethanol sensitivity. *J Pharmacol Exp Ther* 244:866-870, 1988.
- Alpern, H.P., and McIntyre, T.D. Sedative-hypnotic anomalies related to dose of pentobarbital in long-sleep and short-sleep selectively-bred mice. *Pharmacol Biochem Behav* 25:333-336, 1986.
- Bonnichsen, R.K., and Brink, N.G. Liver alcohol dehydrogenase. *Methods Enzymol* 1:495-500, 1955.
- DeFries, J.C. Selective breeding for behavioral and pharmacological responses in laboratory mice. In: Gershon, E.S.; Matthysse, S.; Breakefield, X.O.; and Ciarenello, R.D., eds. *Genetic Research Strategies*

- for *Psychobiology and Psychiatry*. New York: Boxwood Press, 1981. pp. 199-214.
- Deitrich, R.A., and Baker, R.C. Initial sensitivity of rat inbred strains to acute alcohol. *Alcoholism Clin Exp Res* 8:487-490, 1984.
- Deitrich, R.A.; Bludeau, P.; and Baker, R.C. Brain ascorbic acid levels: Mice and rats selected for differences in acute reactions to ethanol. *Alcohol Alcoholism* 23:57-62, 1988.
- Dudek, B.C.; Abbott, M.E.; and Phillips, T.J. Stimulant and depressant properties of sedative-hypnotics in mice selectively bred for differential sensitivity to ethanol. *Psychopharmacology* 82:46-51, 1984.
- Erickson, C.K. Ethanol clearance in nine inbred rat strains. *Alcoholism Clin Exp Res* 8:491-494, 1984.
- Erwin, V.G., and Cornell, K. Effects of 6-hydroxydopamine on brain catecholamines and on acute actions of ethanol in LS/Ibg and SS/Ibg mice. *Alcoholism Clin Exp Res* 10:285-289, 1986.
- Erwin, V.G.; Heston, W.D.W.; McClearn, G.E.; and Deitrich, R.A. Effects of hypnotics on mice genetically selected for sensitivity to ethanol. *Pharm Biochem Behav* 4:679-683, 1976.
- French, T.A.; Masserano, J.M.; Spuhler, K.; Deitrich, R.A.; and Weiner, N. Preliminary characterization of catecholamine systems in rats selectively bred for differential acute sensitivity to alcohol. *Alcoholism Clin Exp Res* 12:307, 1988a.
- French, T.A.; Masserano, J.M.; and Weiner, N. Further studies on the neurochemical mechanisms mediating differences in ethanol sensitivity in LS and SS mice. *Alcoholism Clin Exp Res* 12:215-223, 1988b.
- Hansen, C., and Spuhler, K. Development of the National Institutes of Health Genetically Heterogeneous Rat Stock. *Alcoholism Clin Exp Res* 8:477-479, 1984.

- Hill, W.G. Estimation of realized heritabilities from selection experiments. Selection in one direction. *Biometrics* 30:767-780, 1972.
- Howerton, T.C.; Burch, J.B.; O'Connor, M.F.; Miner, L.L.; and Collins, A.C. A genetic analysis of ethanol, pentobarbital, and methyprylon sleep-time response. *Alcoholism Clin Exp Res* 8:546-550, 1984.
- Knapp, D.R. *Handbook for Analytical Derivatization Reactions*. New York: John Wiley, 1979. p. 639.
- Li, T.-K., and Lumeng, L. Alcohol preference and voluntary alcohol intake of inbred rat strains and the National Institutes of Health heterogeneous stock of rats. *Alcoholism Clin Exp Res* 8:485-486, 1984.
- Litchfield, J.T., and Wilcoxon, F. A simplified method of evaluating dose-effect experiments. *J Pharmacol Exp Ther* 96:99-113, 1949.
- McClearn, G.E., and Kakihana, R. Selective breeding for ethanol sensitivity of short sleep and long sleep mice. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. ADM (81-1113). Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 147-159.
- Palmer, M.R.; Wang, Y.; Fossom, L.H.; and Spuhler, K.P. Genetic correlation of ethanol-induced ataxia and cerebellar Purkinje neuron depression among inbred strains and selected lines of rats. *Alcoholism Clin Exp Res* 11:494-501, 1987.
- Smolen, A.; Marks, M.J.; Smolen, T.N.; and Collins, A.C. Dose and route of administration alter the relative elimination of ethanol by long-sleep and short-sleep mice. *Alcoholism Clin Exp Res* 10:198-204, 1986.
- Spuhler, K., and Deitrich, R.A. Correlative analysis of ethanol-related phenotypes in rat inbred strains. *Alcoholism Clin Exp Res* 8:480-484, 1984.
- Spuhler, K.; Gerhardt, G.; and Palmer, M. CNS monoamine levels and the effects of central noradrenergic lesion on the ethanol-induced sensitivity of long sleep and short sleep mice. *Alcohol* 4:419-424, 1987.

- Spuhler, K.; Hoffer, B.; Weiner, N.; and Palmer, M. Evidence for genetic correlation of hypnotic effects and cerebellar Purkinje neuron depression in response to ethanol in mice. *Pharmacol Biochem Behav* 17:569-578, 1982.
- Tabakoff, B., and Culp, S.G. Studies on tolerance development in inbred and heterogeneous stock National Institutes of Health rats. *Alcoholism Clin Exp Res* 8:495-499, 1984.
- Watson, P.E. Total body water and blood alcohol levels: Updating the fundamentals. In: Crow, K.E. and Batt, R.D. eds. *Human Metabolism of Alcohol*. Vol. 1. Boca Raton, FL: CRC Press, 1989. pp. 41-55.
- Zgombick, J.M., and Erwin, V.G. Central mechanisms of ethanol-induced adrenocortical response in selectively bred lines of mice. *Neuroendocrinology* 46:324-332, 1987.

Alcohol-Stress Interaction: A Genetically Determined Relationship?

Wolfgang H. Vogel, N. Harris, and J. Taylor¹

Introduction

Alcohol (ethanol) use and abuse have been claimed to result from a genetic predisposition of the individual, adverse environmental conditions, or both. Proof for a genetic component in alcohol abuse has been obtained in animals on which drinking behavior and alcohol preference have been clearly shown to be under genetic control (Deitrich and Spuhler 1984). Similarly, selective breeding studies have shown that the effects of alcohol and the response of the body thereto are genetically controlled (Deitrich and Spuhler 1984). In humans, family, twin, and adoption studies have shown a definitive degree of inheritance, and inherited biologic parameters can markedly affect the pharmacokinetics and pharmacodynamics of alcohol (Goodwin 1980).

Among environmental factors and life experiences, adverse social, economic, familial, personal, and professional conditions have been implicated in the initiation and maintenance of alcohol abuse (Cloninger et al. 1981; Rutter 1986). One common factor of many of these environmental conditions has been stress. It has been suggested that the discomfort of stress is relieved by

¹Department of Pharmacology and Department of Psychiatry and Human Behavior, Thomas Jefferson University, Jefferson Medical College, Philadelphia, PA 19107.

alcohol and that this reduction of tension leads to alcohol abuse (alcohol-tension reduction hypothesis) (Capell and Herman 1972).

The genetic aspects of alcohol preference and the response of the organism to this chemical are the main focus of this symposium, and many excellent studies are being presented. A discussion of environmental conditions leading to stress and perhaps alcohol misuse seems actually out of place, since the environment is opposite to genetics. However, evidence is emerging which indicates that individuals are not helplessly manipulated and controlled by stressful life events but that each person responds in a particular reaction called stress to these external or internal challenges and that this response carries a significant genetic component. This paper presents evidence indicating that the stress response is a trait characteristic of an animal, that its modification by alcohol has a genetic component, and that this stress-alcohol interaction may determine to some extent the use or abuse of alcohol by an individual.

Methods and Materials

Male and female rats used in the studies reported belong to the Sprague-Dawley or N/NIH ("Hansen") strains. The stressor used was immobilization; rats were taped by the legs to the laboratory bench for periods of time ranging from 30 to 60 min.

The stress response of the animals was quantitated by measuring the changes of plasma norepinephrine and epinephrine during exposure to immobilization. For this purpose, blood samples were obtained before and during immobilization from an indwelling jugular catheter permanently attached to each animal. This procedure not only is necessary to obtain true values for these plasma catecholamines, but it also allows the monitoring of stress responses of individual animals over time. The same rat can be studied for an entire stress period on one or more occasions. After testing, the animal can be decatheterized and, at a later date, recatheterized and retested. On the basis of their responses to stress, rats can then be chosen for selective breeding and genetic studies.

Plasma catecholamine levels were determined by the radioenzymatic assay procedure marketed by Amersham (Cat a Kit). More specific information on procedural details can be obtained from the references cited for each experiment.

Results

During our stress studies, we noticed that rats showed quite different plasma catecholamine responses to external challenges such as immobilization. Rats were found to be "high," or "low," or "intermediate" responders (DeTurck and Vogel 1980, 1982; Livezey et al. 1985). This differential response was found to be quite consistent for a given rat. Table 1 shows an example of two rats stressed on 2 consecutive days with the identical stressor; different, but for each rat consistent, plasma catecholamine responses can be seen.

Table 1.—Plasma catecholamine response of two male rats exposed to immobilization on 2 consecutive days^a

		Response (pg/ml) at given time (min)			
		0	5	15	30
Catecholamine					
Norepinephrine					
Rat 1					
	Day 1	215	1,900	1,715	1,136
	Day 2	199	2,327	1,897	1,536
Rat 2					
	Day 1	159	617	521	413
	Day 2	201	718	609	387
Epinephrine					
Rat 1					
	Day 1	83	2,320	1,868	1,421
	Day 2	54	2,922	1,815	1,294
Rat 2					
	Day 1	68	817	726	520
	Day 2	75	615	543	463

^aRats were immobilized just after the 0-min (resting baseline) time point.

We also noticed that female rats responded somewhat differently from male rats of the same age and strain (Livezey et al. 1985, 1988). Female rats typically showed somewhat higher and more prolonged stress responses for both norepinephrine and epinephrine (table 2). Thus, a sex difference seems apparent in the catecholamine responses to immobilization.

Table 2.—Plasma catecholamine response of male and female rats exposed to immobilization^a

Catecholamine	Response (pg/ml) at given time (min)			
	0	5	15	30
Norepinephrine				
Males	452±47	1,393±1,215	780±344	527±171
Females	219±70	1,605±919 ^b	1,193±550 ^b	1,196±670 ^b
Epinephrine				
Males	66±99	1,121±1,006	638±297	469±237
Females	79±47	1,439±347	938±415 ^b	774±338 ^b

^aValues are means ± standard deviations for 13-14 male or female rats. Rats were immobilized just after the 0-min (resting baseline) time point.

^b*P*<0.05 as compared with males.

To determine whether the stress response for a particular rat would be consistent for longer periods of time, we stressed a group of rats by immobilization and measured their exact plasma catecholamine responses. After decatheterization and stress-free housing for 1 year, we again catheterized the animals and restressed them in an identical fashion. A comparison of these two stress responses obtained at a younger and an older age showed a general increase in the stress response after aging. Relatively, however, the high responders at the young age were still high responders at the older age, the low responders remained low, and the intermediate responders were still in between the high and low responders. An example (unpublished results) for the consistency of the stress response for five rats is shown in table 3.

Table 3.—Stress responses measured 1 year apart^a

Rat no.	AUC (ng/ml x 30 min)			
	Norepinephrine		Epinephrine	
	Initial	After 1 yr	Initial	After 1 yr
1	17.7	32.0	16.2	47.3
2	21.0	43.0	17.9	58.0
3	23.3	44.3	25.6	63.6
4	37.0	53.2	37.2	67.9
5	42.5	145.1	48.8	170.7

^aAUC is area under the (immobilization) stress curve, a measure of the overall stress response during the 30-min period.

Since environmental conditions were very similar, if not identical, for all rats during these experiments, the consistency of the relative magnitudes of the stress response suggests that the stress response may be a characteristic or trait of a particular rat and therefore carry a genetic component. To obtain information on the genetics of the stress response, pairs of high and pairs of low plasma catecholamine stress responders were bred selectively. At present, we only have the results of the original breeders and the next two generations. Although two generations are not enough to make significant statements, a few trends already seem apparent (unpublished data). Table 4 shows the results for the total area under the stress curve (AUC) for the plasma catecholamine in response to immobilization. As can be seen, the stress responses of both the high and the low male responders are increasing but, as would be expected, the responses of the high responders are increasing the most, and consequently the difference between the low and high responders is becoming greater.

Table 4.—Mean stress responses in selectively bred rats^a

Stressor	Mean AUC (ng/ml x 30 min; <i>n</i> > 20)			
	LRM	HRM	LRF	HRF
Generation 0				
Norepinephrine		21.3		40.3
Epinephrine		28.0		49.0
Generation 1				
Norepinephrine	32.6	38.8	36.7	42.9
Epinephrine	38.0	46.1	49.6	58.8
Generation 2				
Norepinephrine	33.8	41.4	42.7	64.2
Epinephrine	41.8	56.1	91.6	155.0

^aAUC is area under the stress curve, a measure of the overall response during the 30-min (immobilization) stress period. LRM, Low responder, male; HRM, high responder, male; LRF, low responder, female; HRF, high responder, female.

Histograms showing the distributions of responses (AUC) classified according to magnitude for the original breeders and for the next two generations are shown in figures 1 and 2 for male and female rats, respectively. As anticipated, the distributions apparently are becoming wider with each successive generation. Theoretically, continued selective breeding should ultimately produce two completely separate populations.

Although these studies show quite clearly that the stress response is indeed controlled by a genetic component, the stress response is not rigid. That is, single stress response can be modified by environmental conditions, unpleasant experiences, or drugs. For instance, exposure to stressful conditions over longer periods of time has been found to modulate an individual's stress response; this stress response can remain unchanged, or it can increase or decrease over time (Vogel and Jensh 1988). The successive responses of four repeatedly stressed rats are shown in table 5. Although the schedules of chronic restraint were the same for all rats, the animals

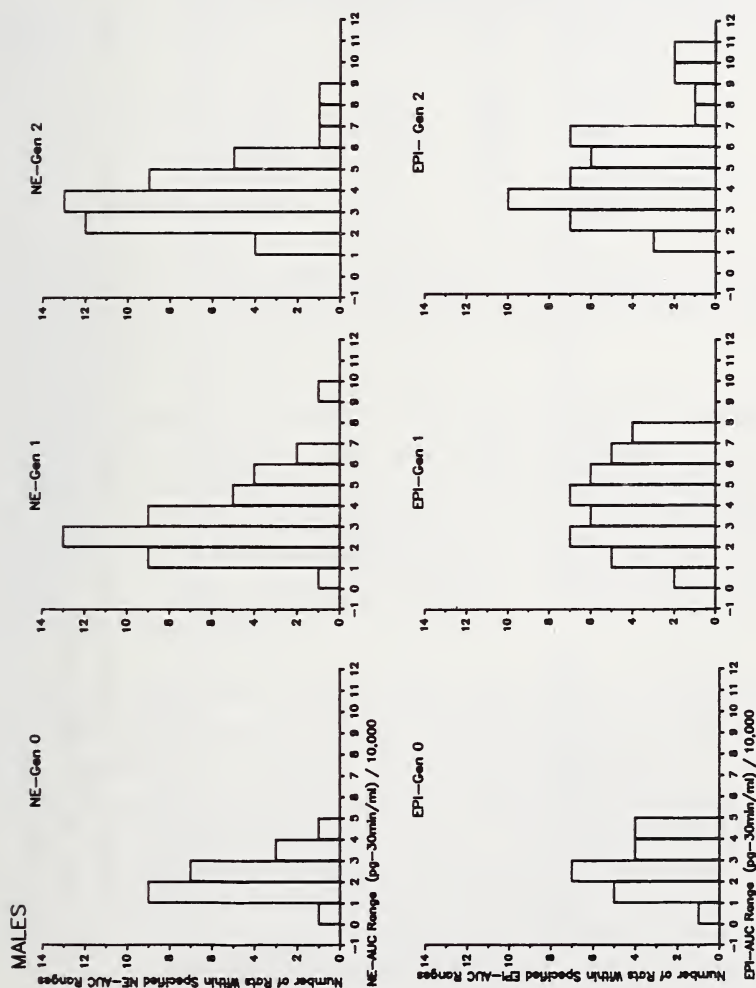


Figure 1.—Frequency distribution of stress responses (AUCs) in selectively bred male rats. Values on y axes are the numbers of rats in each population whose stress responses were within each of the specified ranges.

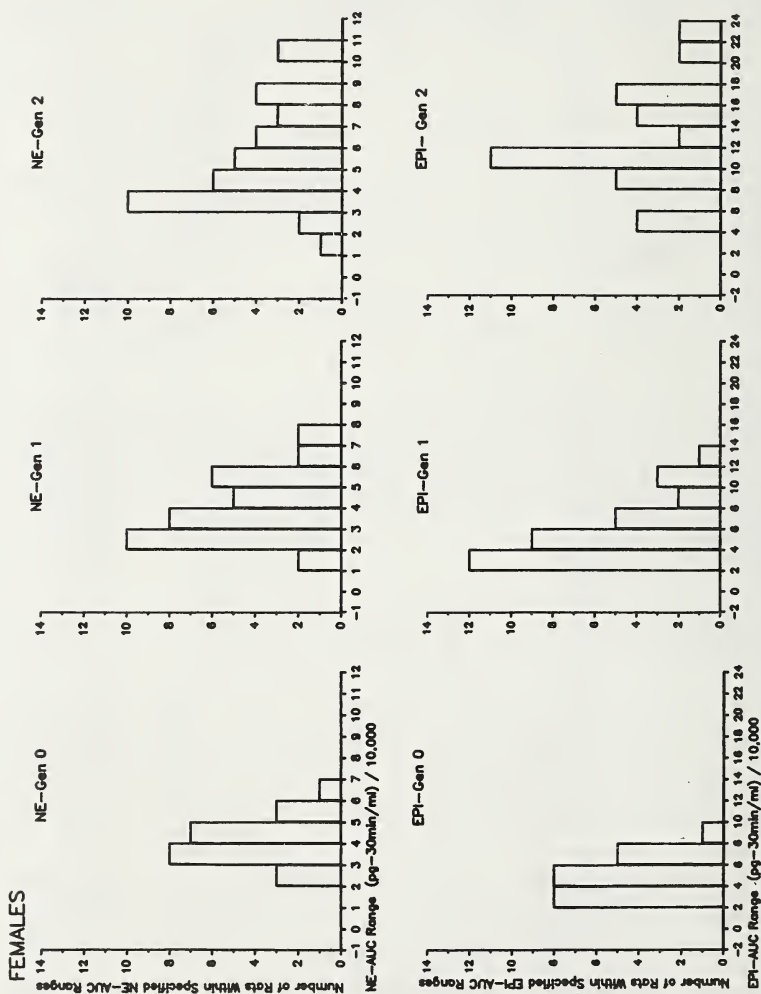


Figure 2.—Frequency distribution of stress responses (AUCs) in selectively bred female rats. Values on y axes are the numbers of rats in each population whose stress responses were within each of the specified ranges.

Table 5.—Effect of daily repeated stress on the plasma norepinephrine response to stress^a

Rat no.	Plasma norepinephrine level (pg/ml) on study day:					
	1	5	10	15	17	31
1	716	476	670	551	779	453
2	346	437	473	496	489	507
3	395	675	662	1,159	1,340	702
4	758	1,203	1,019	1,410	1,658	648

^aRats were immobilized daily until day 17 and then (after a 14-day rest period) on day 31. Blood was collected at a fixed time during the stress on days 1, 5, 10, 15, 17, and 31.

responded quite individually. Rats 1 and 2 seemed to maintain their particular stress responses quite well over time, whereas rats 4 and 5 exhibited gradually increasing magnitudes of response with each successive exposure to restraint. After a rest period of 2 weeks, magnitudes of response reverted to initial stress values (i.e., day 1). Thus, chronicity of adversity changes the stress response, but changes are individualistic and most probably not permanent.

It has been claimed that alcohol reduces stress, is consumed for this reason, and therefore can lead to alcoholism (Capell and Herman 1972). To explore this concept, we measured the plasma catecholamine response to immobilization in rats pretreated with saline or with ethanol (DeTurck and Vogel 1982; Livezey et al. 1988; Vogel et al. 1986). Rats were first stressed without alcohol and, on the next day, stressed under the influence of a small amount of alcohol (1-2 g/kg intraperitoneally). Table 6 shows that alcohol can indeed blunt the plasma catecholamine stress response in rats; the stress response was significantly reduced, although somewhat prolonged, in rats under the influence of alcohol. In nonstressed rats, alcohol had no effect or slightly increased plasma catecholamine levels at the higher dose.

During these studies, we noticed (as is apparent from the large standard variations seen in table 6) marked differences in the reduction of the stress

response by alcohol among our rats. In some rats, alcohol reduced the stress response a great deal, whereas in others it did so only marginally. A comparison of the stress response before alcohol administration with the stress response in rats under the influence of alcohol shows a positive correlation between the pure stress response and the degree of reduction by alcohol (Livezey et al. 1988). In rats with the highest stress response, alcohol reduced the stress response the most; in rats with the smallest stress response, alcohol reduced the stress response the least (table 7). Thus, the magnitude of the response to stress predicts the magnitude of the reduction caused by alcohol.

On the basis of these findings, we conducted a small breeding experiment in which high and low reducers were bred selectively (Vogel and DeTurck 1983). Although only three generations were examined, in the low reducers alcohol did not change the stress response significantly over the generations, whereas in the high reducers alcohol increased the reduction of the stress response substantially (table 8).

Discussion

It is now quite well accepted that the preference for and certain actions of alcohol have a genetic component. Studies on inbred strain differences and heterogeneous animal stocks have shown convincingly that alcohol preference has a marked genetic component; genetically different animals differ in alcohol consumption, and "low" and "high" drinking animals can be distinguished and selectively bred (Deitrich and Spuhler 1984). In addition, there is a large body of evidence showing that the action of alcohol on the body and the response of the body to alcohol are also under genetic control (Deitrich and Spuhler 1984). For instance, rodents differ in the degree of alcohol-induced hypothermia, activity, ataxia, and sleeping time. More recently, these behavioral and physiologic observations have been extended to the molecular level; certain neurotransmitters and transmitter systems have been linked to these observations (Deitrich and Spuhler 1984).

Whereas many of the older studies suffer from methodologic flaws, more recently quite sophisticated experimental procedures have been developed. During this symposium, Karen Spuhler presented an excellent genetic study

Table 6.—Effect of ethanol pretreatment on plasma catecholamine response to stress^a

Stressor	Response (pg/ml) at given time (min)				AUC (ng/mlx30mm)
	0	5	15	30	
Norepinephrine					
Stress	152±47	1,393±1,215	780±344	527±171	20.1±14.1
Alcohol + stress	234±90	475±142 ^b	573±327 ^b	703±609	8.1±6.7 ^b
Epinephrine					
Stress	66±93	1,121±1,006	638±297	469±237	18.2±12.1
Alcohol + stress	119±105	341±157 ^b	392±795 ^b	498±570	6.8±6.4 ^b

^aValues are means ± standard deviations (*n*=15). Rats were immobilized just after the 0-min (resting baseline) time point. AUC is area under the stress curve, a measure of the overall response during the 30-min stress period.

^b*P*<0.05 compared with stress alone.

Table 7.—Correlation between magnitude of stress response and magnitude of reduction of stress response by ethanol^a

Rats tested	Correlation coefficient for (AUC vs. AUC-AUCA)	
	Norepinephrine	Epinephrine
Females (<i>n</i> = 13)	0.7629	0.9813
Males (<i>n</i> = 15)	0.8803	0.9099

^aAUC is the area under the stress curve without alcohol; AUCA is the area under the stress curve when rats received ethanol 15 min before the stress.

Table 8.—Reduction by ethanol of plasma catecholamine stress response in selectively bred rats

Stressor	Stress reduction ^a	
	Breeding for lowest reduction	Breeding for highest reduction
Norepinephrine		
Generation 0	-2.0	+8.3
Generation 1	-2.3	+12.0
Generation 2	-1.4	+20.0
Generation 3	-0.7	+18.5
Epinephrine		
Generation 0	-4.0	+14.8
Generation 1	+0.2	+29.9
Generation 2	-2.2	+22.0
Generation 3	+0.6	+28.4

^aAmounts by which stress responses were reduced by pretreatment with ethanol, i.e., ($AUC_{normal} - AUC_{ethanol}$); AUC is area under the stress curve (nanograms/milliliter x 30 min), a measure of the overall response during the 30-min stress period.

involving replicate lines in "high" and "low" directions with available control lines to show a clear genetic correlation between the hypnotic effect of alcohol and its depression of cerebellar Purkinje neuron activity. Thus, the interaction between alcohol and the response of the individual shows a rather impressive genetic component.

In contrast to genetics are the environmental circumstances and life experiences that also play a role in the use or abuse of alcohol. Here, stressful events and stress have been claimed to play a major role in the etiology of alcoholism and to affect the mental and physical state of the individual. Stress is often viewed as the result of the impact of external or

internal challenges on the organism over which little or no control can be exerted. However, it has been well recognized by psychologists and psychiatrists, although less so by biologists, that individuals show very different responses to the same stressful events or that the stress response is highly individualized (Lazarus and De Longis 1983; Rutter 1986). Thus, the traditional sequence of stressful life events (stressors) causing a behavioral, biochemical, and physiologic response (stress) in an individual that can lead to pathologic damage (health consequences) needs major revision.

Today, the sequence is thought to be quite different, placing the individual in the center of this complex relationship and giving him or her the active and decisive role (Lazarus and De Longis 1983). External or internal events are perceived by the individual who evaluates them in terms of meaning, importance, and coping mechanisms. If the individual decides that the event is not important, the challenge is not great, or the event can be dealt with easily, no biologic changes occur or no stress is experienced; the event becomes nonstressful, and no health consequences will ensue. If, on the other hand, the individual feels that the event is very important or too challenging or believes that he or she cannot cope with it, then biologic changes occur that are experienced as stress. It is the presence of behavioral and biologic changes or stress that now makes the event stressful for this individual. The occurrence of stress raises the possibility of health consequences. If stress is chronic or intense or the individual has weak or diseased organs, pathology or stress-related diseases will occur (Tapp and Natelson 1988). Thus, stress is in the mind of the beholder; we create our own stress, our own stressful events, and our own stress-related diseases.

Since the individual is the originator of the stress response, we must again expect a significant genetic component of this response to environmental challenges. On the basis of psychological studies, the stress response and associated coping mechanisms have indeed been claimed to be rather enduring personality traits (Kobasa et al. 1981). In our studies with plasma catecholamines in rats, we have obtained evidence that the stress response is quite different among animals but seems to be an enduring characteristic for each individual rat. While the stress response may vary and change over time, the rank order under defined housing conditions remains the same. High responders at a young age are also high responders at an older age, whereas young low responders grow to become old low responders.

Intermediate animals will be intermediate at both ages. Preliminary results indicate that rats can be selectively bred for their individual stress responses. The offspring of low responders remained low or increased their stress responses only slightly over the generations, whereas the high responders grew considerably higher in their stress responses with each successive generation, and the difference between both lines increased. Moreover, a frequency distribution plot of the second generation reveals the beginning of a separation of high and low responders. These observations indicate that the stress response as measured in plasma catecholamine changes is a characteristic of a given animal and that this characteristic is under genetic control.

We have also obtained evidence that the stress response can be modified by adverse environmental conditions or by drugs such as alcohol. Again, the observed modification of the stress response depends on the animal. Rats have responded differently but consistently and, in the case of alcohol, can apparently be bred selectively for this response. Thus, not only the initial stress response but also its modification by environmental conditions or alcohol is at least in part genetically controlled. Thus, genes may determine not only how stress is experienced but also whether and how the organism will adapt to the effects of drugs or environmental factors.

How would the findings described above fit into an explanation of alcohol abuse and alcoholism? First, the stress response has been shown to be a characteristic of a particular animal and thus to carry a significant genetic component determining the extent of the stress response. Second, the stress response can be modulated by environmental conditions and individual experiences, but the extent of this modification depends also on the particular animal, which is indicative of a genetic component. Third, alcohol can reduce and antagonize the stress response. Alcohol reduces significantly the stress-induced increases in plasma norepinephrine and epinephrine (DeTurck and Vogel 1980, 1982). It can also reduce stress-induced changes in plasma corticosterone (Brick and Pohorecky 1983) and certain amino acids (Milakofsky and Vogel in press). Alcohol effectively reduces stress-induced increases in heart rate or blood pressure (Graffy Sparrow et al. 1987). In the brain, alcohol antagonizes and increases the stress-induced fall in hypothalamic norepinephrine (DeTurck and Vogel 1982). Similar observations were made with serotonin (Kuriyama et al. 1983). In most

cases, marked individual differences were encountered. In a preliminary breeding study, it was found that this response might also carry a genetic component. Fourth, in all of these actions, alcohol acts quite similarly to anxiolytics such as diazepam and alprazolam (Vogel et al. 1984).

Thus, it can be hypothesized that an individual born with a genetically determined high-stress response, little ability to modify this response through environmental input, and good stress relief by consumption of alcohol would be at high risk for alcohol use and abuse. This individual would also experience high relapse rates after cessation of heavy alcohol use and would probably show little response to psychotherapy and counseling. Indeed, this individual might be a good candidate for anxiolytic therapy, which would provide the same stress reduction without causing the behavioral toxicities of alcohol. An individual with a high stress response, marked stress reduction by alcohol, but an easy modification ability via environmental factors would be at risk for alcoholism but also an excellent candidate for counseling and psychotherapy to prevent or stop alcohol abuse. In contrast, individuals with high stress responses but no stress reduction by alcohol or with low stress responses might not benefit from alcohol at all and might not drink or do so only socially.

Summary

The plasma catecholamine (norepinephrine or epinephrine) stress response and its modulation by environmental conditions and ethanol were studied in male and female rats. Immobilization stress alone caused a quick rise in both catecholamines at the beginning and a decline during the latter part of the stress experience. Animals responded quite differently to this stress but were highly consistent in their individual stress responses. Preliminary data based on a selective breeding study suggest that this stress response has a genetic component and is a characteristic of a particular animal. The stress responses changed in rats repeatedly exposed to stress, and the changes seen were highly individualized; after a rest period, the original stress response returned. A small dose of ethanol reduced the stress response; again, reductions were quite different among rats and highly individualized. Preliminary results of a breeding study show that the extent of the reduction of the stress response by ethanol carries a genetic component. These results

indicate that the response of an animal to an environmental challenge and its modification by ethanol are genetically influenced. It can be speculated that stress reduction by alcohol may play a role in the etiology of alcoholism and that the stress response and its reduction are genetically controlled.

References

- Brick, J., and Pohorecky, L.A. The neuroendocrine response to stress and the effect of ethanol. In: Pohorecky, L.A., and Brick, J., eds. *Stress and Alcohol Use*. New York: Elsevier, 1983. pp. 389-402.
- Capell, H., and Herman, P. Alcohol and tension reduction. *Q J Studies Alcohol* 33:33-64, 1972.
- Cloninger, C.R.; Bohman, M.; and Sigvardsson, S. Inheritance of alcohol abuse. *Arch Gen Psychiatry* 38:861-868, 1981.
- Deitrich, R.A., and Spuhler, K. Genetics of alcoholism and alcohol actions. In: Smart, R.G.; Capell, H.D.; Glaser, F.B.; Israel, Y.; Kalant, H.; Popham, R.E.; Schmidt, W.; and Sellers, E.M., eds. *Research Advances in Alcohol and Drug Problems*. Vol. 8. New York: Plenum Press, 1984. pp. 47-98.
- DeTurck, K.H., and Vogel, W.H. Factors influencing plasma catecholamine levels in rats during immobilization. *Pharmacol Biochem Behav* 13:129-131, 1980.
- DeTurck, K.H., and Vogel, W.H. Effects of acute ethanol on plasma and brain catecholamine levels in stress and unstressed rats: Evidence for an ethanol-stress interaction. *Pharmacol Exp Ther* 223:348-254, 1982.
- Goodwin, D.W. The genetics of alcoholism. *Subst Alcohol Actions Misuse* 1:101-117, 1980.
- Graffy Sparrow, M.; Roggendorf, H.; and Vogel, W.H. Effect of ethanol on heart rate and blood pressure in nonstressed and stressed rats. *Life Sci* 40:2251-2559, 1987.

- Kobasa, S.C.; Maddi, W.R.; and Courington, S. Personality and constitution as mediators in the stress-illness relationship. *J Health Serv Behav* 22:368-378, 1981.
- Kuriyama, K.; Kanmori, K.; and Yoneda, Y. Preventive effect of alcohol on stress-induced alterations in metabolism and function of biogenic amines and gamma-aminobutyric acid (GABA) in neuroendocrine system. In: Pohorecky, L.A., and Brick, J., eds. *Stress and Alcohol Use*. New York: Elsevier, 1983. pp. 403-420.
- Lazarus, R.S., and De Longis, A. Psychological stress and coping in aging. *Am Psychol* March:245-254, 1983.
- Livezey, G.T.; Balabkins, N.; and Vogel, W.H. The effect of ethanol (alcohol) and stress on plasma catecholamine levels in individual female and male rats. *Neuropsychobiology* 17:193-198, 1988.
- Livezey, G.T.; Miller, J.M.; and Vogel, W.H. Plasma norepinephrine, epinephrine and corticosterone stress responses to restraint in individual male and female rats, and their correlation. *Neurosci Lett* 62:51-56, 1985.
- Milakofsky, L., and Vogel, W.H. Effect of ethanol on plasma amino acids in stressed rats. (In press.)
- Rutter, M. Meyerian psychobiology, personality development and the role of life experiences. *Psychiatry* 143:1077-1087, 1986.
- Tapp, W.N., and Natelson, B. Consequences of stress: A multiplicative function of health status. *FASEB J* 2:2268-2271, 1988.
- Vogel, W.H., and DeTurck, K.H. Effects of alcohol on plasma and brain catecholamine levels in stressed and unstressed rats. In: Pohorecky, L.A., and Brick, J., eds. *Stress and Alcohol Use*. New York: Elsevier, 1983. pp. 429-438.
- Vogel, W.H.; DeTruck, K.; and Miller, J.M. Differential effects of ethanol on plasma catecholamine levels in rats. *Biochem Pharmacol* 22:3983-3987, 1986.

Vogel, W.H., and Jensh, R. Chronic stress and plasma catecholamine and corticosterone levels in male rats. *Neurosci Lett* 87:183-188, 1988.

Vogel, W.H.; Miller, J.M.; DeTurck, K.H.; and Routzhan, B.K. Effects of psychoactive drugs on plasma catecholamines during stress in rats. *Neuropharmacology* 23:1105-1107, 1984.

Discussion

Q: Are you measuring corticosterone as well as catecholamines?

VOGEL: Yes we are measuring this. In some rats corticosterone goes up very much and in others only a little. But high responders on catecholamines are not always high responders on corticosterone, nor are low responders on corticosterone always low responders on catecholamines. On the other hand, a high norepinephrine responder is usually a high epinephrine responder and vice versa.

ERWIN: What was the dose of ethanol and how was it administered?

VOGEL: The dose was 1 gm/kg given IP.

ERWIN: In the SS and LS mice if you give 1 gm/kg ethanol IP you see marked increased plasma catecholamines. Thus I would expect that such a dose given to rats would be sufficient stressor to actually increase catecholamines, yet in one slide it appeared that was not the case.

VOGEL: You are correct in that. If you read the literature you see ethanol referred to as a stressor. Certainly if you give more than 2 gm/kg, an increase in catecholamines is seen. The other aspect is that if you give ethanol in a concentration of more than 20% that is very irritating and of course that will raise the levels of catecholamines.

SPUHLER: The dose that we use currently is 3 gm/kg administered in a 15% solution. We have also looked at sex difference for initial sensitivity and

we find that the males are more sensitive than the females but the extent of this difference is the same across the lines.

VOGEL: We find a difference in that females respond much higher and longer than the males.

SPUHLER: We did measure realized heritability and found that the average value was 26% for all the lines.

VOGEL: I would also like to offer our animals for use by any other investigators.

P. ERIKSSON: In our drinker and nondrinker rats we have noticed that the nondrinkers are much more stressed. Another question, however, is that when one gives high doses of ethanol the animal loses the righting reflex. What is there to be stressful when this happens?

VOGEL: The definition of stress is any change in any physiological, behavioral or biochemical parameter. Any change from normal is a stress. You can measure a variety of things such as plasma catecholamines, corticosterol, heart rate to assess this change. However, in your case, it is not stress but sheer intoxication, etc. and they will all change.

Selective Breeding for Two Measures of Sensitivity to Ethanol¹

*John C. Crabbe^{2,3,4}, Daniel J. Feller,^{2,4}
and Tamara J. Phillips^{2,3}*

Introduction

One tool for exploring the genetic determinants of sensitivity to ethanol (EtOH) that has gained popularity is the development of genetically selected lines of rats and mice. Since a 1978 NIAAA-sponsored conference reviewed such lines (McClearn et al. 1981), several new lines have been developed. The purpose of this paper is to review progress in two projects that we have been conducting in Portland. We chose to model two different effects of EtOH. In one set of lines, we are developing a genetic animal model for sensitivity to a depressant effect of EtOH, acute hypothermia (HT). These lines are designated HOT (unresponsive) and COLD (responsive). The

¹These studies were supported by a grant from the Veterans Administration and by PHS-ADAMHA-NIAAA grants AA05828, AA06243, and AA06498. We thank David Baxter, Janet Dorow, Sue Burkhart-Kasch, Cathy Merrill, Erik Terdal, and Emmett Young for performing these studies.

²Research Service, VA Medical Center, Portland, OR 97201.

³Department of Medical Psychology, Oregon Health Sciences University, Portland, OR.

⁴Department of Pharmacology, Oregon Health Sciences University, Portland, OR.

other set of lines is being selected for EtOH-stimulated locomotor activity in an open field (ACT), since this behavioral response may model the disinhibitory and/or euphoric effects of the drug. FAST mice are highly stimulated by EtOH, and SLOW mice show less activation or even depressed activity after EtOH administration. Progress will be characterized genetically, and the emergence of correlated responses to selection will also be discussed.

Husbandry

Mice are housed two to six in each Plexiglas cage, with sawdust bedding changed twice weekly under laminar-flow conditions. A light-dark cycle of 0600-1800 light is maintained; behavioral testing is conducted between hr 3 and 8 of the light period. Colony and testing room temperature is maintained at 24 ± 1 °C. Animals have ad libitum access to food and water.

Selection Method

An initial population of 20 breeding pairs of HS/Ibg mice was established. These animals are genetically heterogeneous and were derived from crossing eight inbred strains. By using standard techniques for within-family selective breeding, a responsive and an unresponsive line are propagated (Crabbe et al. 1985). In addition, a randomly selected control line, designated CON, is maintained. Each experiment is replicated, so we are developing two lines responsive and two lines unresponsive to each effect of EtOH. The two CON lines are held in common for both selections. Each line comprises nine families.

Hot and Cold Mice

Testing Procedure

The general procedures we use for HT testing have been published elsewhere (Crabbe et al. 1982). A baseline temperature is taken, and each mouse is weighed and injected intraperitoneally (IP) with EtOH (3 g/kg, 20 percent [vol/vol] in physiologic saline) and is returned to its cage. At 30 and

60 min after injection of EtOH, the test temperature of each mouse is measured. Immediately after the 60-min temperature assessment, a 10- μ l blood sample is taken for blood EtOH concentration (BEC) determinations. The maximal change from baseline is used to index sensitivity to EtOH to select parents for mating in the COLD lines, and the minimum change is used for this purpose in the HOT lines. Reasons for choosing change scores over alternative indices of HT responsiveness have been discussed elsewhere (Crabbe and Weigel 1987).

Response to Selection

Each generation, both COLD and both HOT lines are tested under identical environmental conditions, so it is possible to compare the selected lines directly. The success of selective breeding in increasing the HT response to EtOH in the COLD lines and decreasing it in the HOT lines is shown in figure 1, where HT response at 60 min in COLD, HOT, and CON lines is plotted as a function of generation of selection pressure. CON lines are tested only each third generation. A preliminary report of results from this selection has been published (Crabbe et al. 1987a). Differences between replicate pairs of the selected lines are relatively minor, being limited to the fact that COLD-1 mice become more hypothermic than COLD-2 mice, whereas HOT-1 and HOT-2 mice do not differ. Thus, future experiments on thermoregulatory variables with these mice can safely disregard the difference between replicate lines, although both remain available for confirmation of genetic correlation between other traits and EtOH-induced HT. In many of the studies of correlated responses to selection discussed below, we ignore differences between replicate COLD and HOT lines, mentioning them only when replicates differ significantly. Although sex differences in HT sensitivity were present, the pattern of results suggested that they do not importantly interact with the genetic predisposition to EtOH-induced HT: sex differences were similar in HOT and COLD mice but were more pronounced in the nonselected CON line.

Estimation of Genetic Parameters

Discussion of the various genetic parameters characterizing this selection has been presented elsewhere (Phillips et al. submitted). Heritabilities (h^2) for

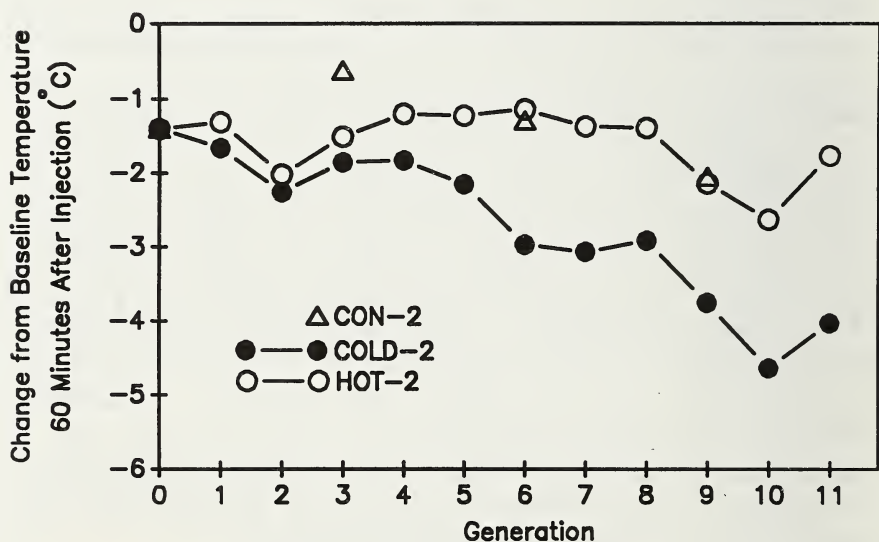
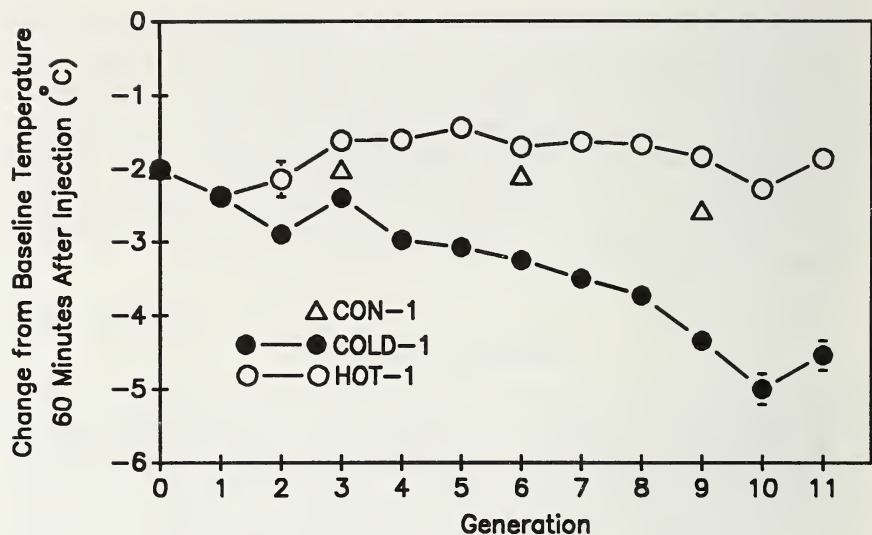


Figure 1.—Mean \pm SE decrease from baseline temperature 60 min after injection of 3 g/kg of EtOH in HOT, COLD, and nonselected CON mice over generations of selective breeding. (Top) First replicate set of lines; (bottom) second replicate set of lines. CON mice were tested only in generations S_0 , S_3 , S_6 , and S_9 . Most SE bars are obscured by symbol.

the total realized response difference between HOT and COLD lines within each replicate were estimated by standard methods (Hill 1972*a,b*; Falconer 1983). The combined estimate of total realized heritability was $h^2 = 0.17$ after five generations of selection. This means that 17 percent of the variance in EtOH-induced HT in mice is presumed to be of additive genetic origin. The inbreeding coefficient (F) was computed according to the method of Falconer (1983) and continues to increase at near the expected rate of 1.5 percent per generation. Trait-irrelevant inbreeding may be assumed to play a relatively minor role in determining differences between COLD and HOT lines.

Dose-Effect Relationships and Ethanol Metabolism

Periodically during the course of selection, we have assessed the dose dependence of the response to EtOH-induced HT in COLD and HOT mice. Table 1 shows the results from such an experiment in which mice from both replicates of the 11th selected generation (S_{11}) were given doses of 1, 2, 3, or 4 g/kg of EtOH after baseline temperature measurements (Crabbe et al. 1988*b*). Temperatures 30 min post-EtOH are shown as a difference from baseline temperatures. COLD mice had greater HT responses than HOT mice, and the dose dependence of EtOH-induced HT was confirmed in all lines. There were significant differences between lines at all doses except 1 g/kg. Mice from the first replicate set of lines had greater HT than did mice from the second replicate set, but there were no interactions with dose or line. Interestingly, the magnitude of the difference seen at the lowest effective dose tested (2 g/kg) was less than at higher doses. A possible explanation for this phenomenon is that challenge of the set of thermoregulatory control mechanisms by EtOH is hierarchical. If relatively low doses of EtOH or levels of HT engage only a subset of effector mechanisms to reestablish normal body temperature, and relatively high doses of EtOH or levels of HT engage additional effector systems, COLD and HOT mice might have developed differences only in the latter. Similarly, the current uncertainty about whether EtOH induces regulated (changes in set point) or unregulated (poikilothermic) temperature changes could be resolved if COLD and HOT mice were found to differ in mechanism of

Table 1.—Dose-response analysis of hypothermic sensitivity in HOT and COLD mice

Line	Dose response at EtOH dose (g/kg) of ^a :			
	1	2	3	4
COLD-1	-0.3±0.4	2.2±0.4	3.4±0.3	3.1±0.6
HOT-1	-0.2±0.1	1.8±0.6	2.3±0.3	2.5±0.3
COLD-2	-0.2±0.2	1.9±0.4	3.0±0.5	3.2±0.3
HOT-2	-0.4±0.2	0.2±0.5	1.0±0.5	1.8±0.2

^aMean ± SE reduction from baseline temperature (°C) 30 min after injection of 3 g/kg of EtOH in groups of five to seven mice.

EtOH action on thermoregulation (Kalant and Le 1984). Studies of the effects of EtOH on thermal preference and physiologic systems will need to be performed to characterize the difference between COLD and HOT mice in HT sensitivity to EtOH more completely.

It is possible that COLD and HOT lines are differing in response to EtOH because of differences in pharmacokinetic response to EtOH. If COLD lines were to achieve higher BEC than HOT lines after acute EtOH, they would be expected to differ in HT response even if they did not differ in central nervous system (CNS) sensitivity to EtOH. Almost all studies have shown that HOT and COLD mice do not differ significantly in BEC at 60 min after injection of a fixed dose of EtOH (Crabbe et al. 1987a, 1988b, Feller and Crabbe submitted b). We assessed the rate of metabolism after a 3-g/kg dose of EtOH in mice from S₁₀. Mice from each line and replicate combination were sacrificed at 1, 2, 3, or 4 hr post-EtOH, and brains were assayed for EtOH concentration. Brain EtOH concentrations (BrEC) 1-4 hr after injection declined linearly over time, and lines differed significantly. The line difference resulted from an average BrEC (over all doses) of 2.01 mg/g of brain in the HOT mice and 2.22 mg/g of brain in the COLD mice. HOT mice had nonsignificantly lower BrEC than COLD mice only at 3 and 4 hr postinjection. COLD mice were found to metabolize EtOH at 0.86 mg/g of brain/hr, whereas HOT mice metabolized EtOH at 1.01 mg/g of brain/hr. Zero-time concentrations of EtOH for each line were 4.37±0.37 and

4.53 \pm 0.40 mg/g of brain for the COLD and HOT lines, respectively (mean \pm standard deviation), a difference that is not significant. Since doses were administered as grams per kilogram of body weight and body weights did not differ, this finding suggests that volumes of distribution in the two lines are not different. In summary, it appears that there may be a small difference in metabolic rate between the lines, with the HOT mice metabolizing EtOH more quickly. This difference may be limited to higher doses of EtOH and may be secondary to the line differences in body temperature at these doses (Crabbe et al. in 1988b).

Generalization to Other Alcohols and Depressants

In a series of experiments, we sought to see whether selection for EtOH HT had effected change in sensitivity to other depressants. Figure 2 shows that COLD mice were more sensitive than HOT mice to several straight-chain alcohols after seven generations of selection. Similar response differences were seen after administration of pentobarbital, phenobarbital, the water-soluble depressant methyprylon, the highly lipid-soluble alcohol ethchlorvynol, and diazepam. These experiments demonstrated that genes controlling EtOH HT also control HT responses to other depressants, suggesting commonality in their mechanism of action on the thermoregulatory system (Feller and Crabbe submitted *b*). As discussed below, generalization was not complete, since there were no differences between HOT and COLD mice in response to some other drugs affecting specific neurotransmitter systems.

As selection pressure was exerted each generation, the magnitude of the difference between COLD and HOT mice increased (figure 1). We repeated the tests of sensitivity to other depressant agents described above in mice of S_{11} and S_{12} . In general, we found that the magnitude of the line difference in these correlated responses to selection was also augmented by additional selection (Feller and Crabbe submitted *b*). Parallel development of a correlated response to selection in replicated selected lines offers very strong evidence for a true genetic correlation (Belknap et al. 1989; Horowitz and Dudek 1983). One interesting example of this phenomenon is given in table 2. The peripheral vasodilator hydralazine, when tested in S_7 mice, did not produce differential HT in HOT and COLD mice. When the compound was

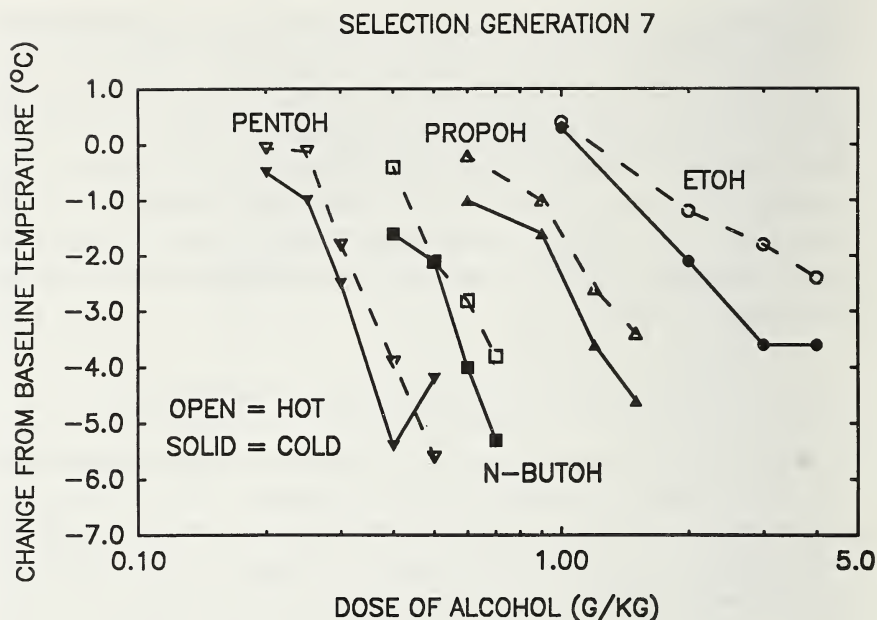


Figure 2.—Mean maximum decrease from baseline temperature in groups of 12 HOT and COLD mice from S_7 30 or 60 min after injection of different doses of straight-chain alcohols. Mice from first and second replicates are pooled for each drug. EtOH (ethanol), PROPOH (*n*-propanol), N-BUTOH (*n*-butanol), PENTOH (*n*-pentanol).

retested in S_{11} mice, a significant difference was present. In S_7 , we concluded that the differences between HOT and COLD mice were largely due to CNS sensitivity (Crabbe et al. 1987a), but it now appears that peripheral thermoregulatory mechanisms may have been recruited into the difference between HOT and COLD mice (Feller and Crabbe submitted a).

Tolerance

We were naturally curious to know whether HOT and COLD mice would differ in development of tolerance to EtOH HT. We administered 3 g/kg of EtOH or saline daily for 4 days and tested for HT after giving all mice EtOH

on day 5. Neither line developed a significant tolerance when tested at 25 °C. We repeated this experiment at an ambient temperature of 18 °C. to increase the magnitude of initial HT. The HT response of COLD mice was significantly attenuated by previous EtOH injections, whereas that of HOT mice remained approximately the same (table 3). Even though mice were equally exposed to EtOH, COLD mice had significantly greater HT than did HOT mice during the 4 days of EtOH exposure to induce tolerance. In addition, HOT mice might have metabolized EtOH more rapidly than did COLD mice. Both of these effects would tend to reduce the duration of exposure of HOT mice to HT relative to COLD mice during repeated injections of EtOH.

Because degree of tolerance to EtOH HT could be thought to reflect magnitude and duration of HT, we sought to equate the HT experience of the two lines in a final experiment. HOT mice were given 4 g/kg and COLD mice were given 2 g/kg during both tolerance acquisition and testing. Three prior injections of EtOH induced tolerance in COLD mice but a tendency toward enhanced HT response in HOT mice (data not shown). Thus, the failure of HOT mice to develop tolerance is not attributable to insufficient exposure to EtOH HT. In all of these experiments, BEC at the time of testing was assayed and found not to differ. We conclude that the differences

Table 2.—Change in sensitivity to hydralazine HT during selection in HOT and COLD mice

Line	Change in sensitivity in ^a :	
	S ₇	S ₁₁
COLD-1	2.7±0.5	4.4±0.4
HOT-1	2.1±0.4	2.2±0.5
COLD-2	3.0±0.6	4.2±0.3
HOT-2	2.6±0.2	2.8±0.4

^aMean ± SE reduction from baseline temperature (°C) 30 min after injection of 3.2 mg/kg of hydralazine in groups of 6-8 mice after 7 or 11 generations of selective breeding for sensitivity to EtOH HT.

Table 3.—Development of tolerance to EtOH HT in HOT and COLD mice

Line	Determination after given no. of previous EtOH administrations ^a	
	0	4
COLD	4.3±0.4	3.1±0.4
HOT	2.4±0.3	2.3±0.3

^aMean ± SE reduction from baseline temperature (°C) 60 min after injection of 3 g/kg of EtOH in groups of 14-15 mice. Replicate lines are combined.

between lines are in functional tolerance rather than due to metabolic factors (Crabbe et al. 1988b).

HT sensitivity and tolerance are genetically correlated (Crabbe et al. 1982). If the thermoregulatory effector mechanisms elicited by EtOH in HOT mice are different from (or a subset of) those elicited in COLD mice, this fact could imply that the lowest hierarchical level of effectors does not develop tolerance upon repeated EtOH administration, whereas tolerance does develop in the remaining systems. This view is consistent with the developing characterization of tolerance to EtOH as a collection of effect-specific phenomena rather than a monolithic adaptation to the chronic presence of the drug.

Neuropharmacologic Analyses

Another group of experiments examined the possibility that specific neural transmitter systems subserve the differences in HT sensitivity to EtOH between HOT and COLD mice. These experiments (summarized in table 4) administered agonists and specific receptor antagonists for dopaminergic and α_1 -adrenergic systems and examined their effects on HT in the absence or presence of EtOH. Although the agents studied produced the expected

Table 4.—Summary of effects of dopaminergic and α -adrenergic drugs on body temperature and EtOH HT in HOT and COLD mice^a

Drug	Mechanism	Treatment
Apomorphine	Dopamine agonist	Drug only Drug + EtOH
Chlorpromazine	Dopamine antagonist	Drug only
Quinpirole	Dopamine D ₂ agonist	Drug only Drug + EtOH
SKF 38393	Dopamine D ₁ agonist	Drug only Drug + EtOH
ST 587	α_1 -adrenergic agonist	Drug only Drug + EtOH

^aDrugs were given in multiple doses alone or versus 2 g/kg of EtOH (3 g/kg for ST 587). HOT and COLD mice responded equally to all drugs except EtOH.

effects on body temperature, the HOT and COLD mice responded similarly to all tested agents (Feller and Crabbe submitted *a*). Thus, we cannot at this time identify a particular neurochemical substrate for the differences in HT sensitivity to EtOH between HOT and COLD mice.

Other Correlated Responses to Selection

Withdrawal was also tested in HOT and COLD mice. Earlier experiments with a number of inbred strains of mice had demonstrated an apparent negative genetic correlation between sensitivity to the initial hypothermic effects of EtOH and the severity of EtOH withdrawal after cessation of forced inhalation (Crabbe et al. 1983*b*). When HOT and COLD mice were exposed to identical regimens of forced EtOH exposure for 72 hr and withdrawn, there was a large difference (more than twofold) between HOT-1 and COLD-1 mice in handling-induced convulsion severity during withdrawal. However, HOT-2 and COLD-2 mice did not differ (Crabbe et al. 1988*b*). The magnitude of difference in the first replicate set of HOT and

COLD lines could represent the effects of a single locus: significant single-locus influence was suggested earlier in a panel of recombinant inbred strains derived from the cross of C57BL/6 and DBA/2 parental inbred strains (Crabbe et al. 1983a).

HOT and COLD mice were also tested for responsiveness to the activating effects of EtOH in an open field. Mice were tested twice for 4 min, starting 2 min after IP injection of EtOH (day 1) and saline (day 2). HOT-1 and COLD-1 mice did not differ in activity, but HOT-2 mice showed seven- to eightfold more activation than did COLD-2 mice and two- to threefold more activation than did either COLD-1 or HOT-1 mice (Crabbe et al. 1988b). These results are also suggestive of the influence of a single locus on EtOH ACT, a possibility also raised by results in the selection study discussed below.

Finally, HOT and COLD mice were tested for the development of a taste aversion conditioned by EtOH. Mice were allowed to drink sodium saccharin solutions and then injected with EtOH. On the following day, access to water only was offered, and no injections were given. The next day, access to the highly preferred saccharin solution was again followed by EtOH injection. This alternating pattern of conditioning days and water days led to a gradual reduction in saccharin intake. HOT mice of both replicates developed more pronounced conditioned taste aversion than did COLD mice (C.L. Cunningham, C.L. Hallett, and L. North unpublished data).

Summary

Results with the HOT and COLD selected lines provide further evidence that sensitivity to the effect of EtOH on thermoregulation in mice is under substantial genetic control. Since a substantial difference in sensitivity to EtOH HT exists in both genetic replicates and has increased over generations of selection pressure, it may be argued strongly that genuine genetic effects rather than nonspecific effects of inbreeding determine the differences. Results to date also confirm the genetic correlation between sensitivity and tolerance to EtOH HT that was originally identified in studies with inbred strains of mice. They suggest that other behavioral responses to EtOH, such as withdrawal severity and locomotor ACT, may share some genetic substrate in common with EtOH-induced HT, but such common

genetic determination does not appear to be substantial. The HOT and COLD selected lines should be a useful genetic animal model for future experiments designed to explore the mechanisms underlying hypothermic sensitivity and tolerance to EtOH.

Fast and Slow Mice

Testing Procedure

The general procedures we use for ACT testing have been published elsewhere (Crabbe et al. 1987b). Mice are tested in the colony room on 2 successive days at a 24-hr intertest interval. At 2 min after injection, each mouse is placed in the middle of one of two Lehigh Valley open fields (61 cm in diameter), and photocell beam interruptions are counted. On one test day, basal activity is assessed after administering the mouse a saline injection. On the other test day, each mouse is given an injection of ethanol and tested as described. Testing for generations S_0 - S_5 was in the order saline-EtOH (1.5 g/kg) under dim ambient lighting conditions. For reasons to be elaborated below, testing parameters beginning with generation S_6 were changed to the order EtOH-saline, with an EtOH dose of 2.0 g/kg, under bright light. The EtOH concentration was always 20 percent (vol/vol).

Response to Selection

FAST and SLOW lines do not differ in response to saline. Mean ACT scores (EtOH-saline) for each line and replication are shown in figure 3 through generation S_{11} . There was an evident divergence between each pair of FAST and SLOW lines in S_1 after only one generation of selection. For reasons elaborated below, we chose to test the S_6 and subsequent generations under different conditions. Following this change in test parameters, there was again an increase in divergence between S_6 and S_7 . When last tested, the CON-2 line was approximately intermediate to the FAST-2 and SLOW-2 lines, whereas the CON-1 line closely resembled the SLOW-1 line.

We conclude that the result of selective breeding has been to generate mouse lines that differ significantly in response to EtOH-induced open-field activity.

Selective Breeding for Initial Sensitivity

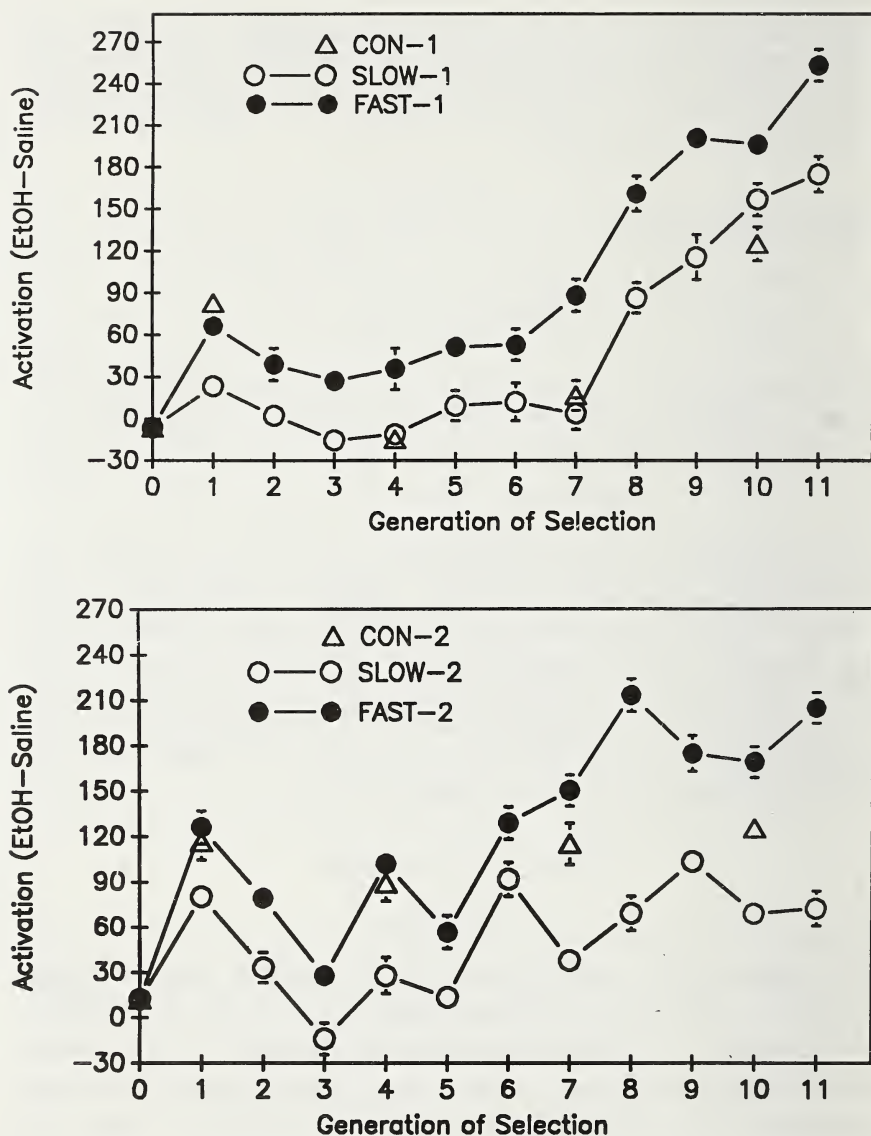


Figure 3.—Means \pm SE ACT scores (EtOH-saline) for FAST, SLOW and nonselected CON mice over generations of selective breeding. (*Top*) First replicate set of lines; (*bottom*) second replicate set of lines. CON mice were tested only in generations S_0 , S_1 , S_4 , S_7 , and S_{10} . Most SE bars are obscured by symbol.

The effect of environmental factors unrelated to selection can be seen in figure 3. In the first selected generation, for example, mice responded to EtOH on the average with relatively more stimulation than in generation 3, in which all lines tested had low ACT scores. Such differences are due to some unexplained change (e.g., seasonal) in the environment affecting all genotypes. An environmental difference of greater consequence can be seen by comparing the results of generations S_6 - S_{11} with those of previous generations. Greater ACT scores were apparent in both replicates but particularly in the first replicate lines. The source of this environmental change may be largely attributed to the change to different testing parameters in generation S_6 .

Estimation of Genetic Parameters

Examination of figure 3 shows that the interpretation of estimates of realized heritability would be difficult, given the change in selection criterion in S_6 . Total realized heritability for the diverging response (i.e., the difference between ACT [EtOH-saline] in the FAST and SLOW lines) was estimated separately in the two replicates after S_6 . Using several different sets of assumptions, estimates were between 0.25 and 0.67. A more detailed discussion of heritability in this selection is given elsewhere (Phillips et al. submitted). Inbreeding was estimated as described by Falconer (1983) and increased at the average rate (across all six lines) of just under 2 percent per generation, consonant with our earlier experience with this breeding scheme (Crabbe et al. 1985). It seems safe to conclude that there is a significant heritable component to this response and that the FAST and SLOW lines do not suffer unduly from inbreeding unrelated to selection.

Environmental Dependence of Selection Response

In the first generation of selection, response to selective pressure appeared to develop in both replicates. However, four additional generations of selection did not reward us with increased divergence. There are several possible reasons for this. First, there could be inadequate additive genetic variance underlying the trait. However, most evidence suggests that this is not the case. Second, there could have been inadequate selection differential, but S

averaged 70 ACT (EtOH-saline) counts in the FAST lines and xx counts in the SLOW lines over the first six selected generations in the four lines and has not declined over generations. We therefore rejected this explanation. Third, the observed pattern could be explained by the influence of a single gene determining the response. This remains a distinct possibility, and the large difference in ACT seen in one replicate of the HOT and COLD selected lines also supports this possibility. Another possibility is there is a "signal-to-noise" problem. The stimulant effect of EtOH on ACT in mice is extremely variable. Some of this variability is genotypic (Crabbe 1983, 1986), but Reed (1977) estimated the heritability as only $h^2=0.12$ for EtOH-stimulated open-field activity in mice tested under conditions similar to those we used in S_1 - S_5 .

In view of these considerations, we undertook several experiments to identify the effect of various environmental and procedural manipulations on the ACT response. We tested FAST and SLOW mice to see whether reversal of drug test order affected the magnitude of the difference in response between lines. We also assessed the role of lighting condition on the magnitude of the genetic difference between lines. Groups of FAST and SLOW mice were tested twice in an open field. One group in each line and replicate received EtOH on day 1, and the other received saline. Each group received the other drug on test day 2. EtOH was administered 20 percent (vol/vol) by IP injection, and testing was between 2 and 6 min after injection. Testing was under dim- or bright-light conditions. We found that the magnitude of the difference between FAST and SLOW mice was greater under bright light than under dim light. The line difference was also greater when tested in the EtOH-saline order, and this increase in magnitude was not due to a line difference in decline of saline scores (Crabbe et al. 1988). Starting with generation S_6 , we changed the selection procedure to optimize the genetic differences just described and increased the EtOH dose to 2.0 g/kg. We tested the reliability of the current selection index in FAST and SLOW mice of generation S_6 - S_7 and found the test- retest correlation of two ACT scores obtained 6 days apart to be $r = 0.55$. Finally, we have tested FAST and SLOW mice in other open-field apparatus, using both repeated-testing (within-animal) and between-group designs and have found the FAST-versus-SLOW differences robust across different circumstances.

Dose-Effect Relationships and Ethanol Metabolism

In an experiment with mice from S_8 - S_9 , different doses of EtOH were given and mice were monitored for activity in an open field. Data were collected for each 5-min block of time for 30 min postinjection. Table 5 shows the dose-response function for mice of the FAST and SLOW lines (combined replicates) 5 min after injection. The differences between lines were most marked at lower doses. Furthermore, time-response analyses showed that FAST mice were consistently more stimulated than SLOW mice but that the differences were most marked early after injection, which corresponds to the time period examined for the selection criterion.

In several experiments, BrEC or BEC was determined after acute EtOH injections or chronic administration, BrEC and BEC 6 min after injection of the selection dose (2 g/kg) revealed no differences between FAST and SLOW mice of either replicate from S_6 - S_7 . Similarly, the selected lines did not differ in rate of clearance from blood of a 2-g/kg EtOH dose. We conclude that selection for EtOH-induced ACT has not altered parameters of EtOH metabolism.

Table 5.—Dose-response analysis of EtOH ACT in FAST and SLOW mice

Dose (g/kg)	ACT score ^a	
	FAST	SLOW
0	182 ± 14	200 ± 11
1.0	272 ± 15	216 ± 16
1.5	324 ± 18	238 ± 17
2.0	285 ± 20	213 ± 15
2.5	276 ± 14	247 ± 12
3.0	202 ± 16	230 ± 20

^aMean ± SE ACT score (EtOH-saline) for groups of approximately 23 FAST and SLOW mice tested for 5 min after injection in an open field.

EtOH-induced Ataxia

We were interested in the possibility that FAST and SLOW lines might differ in ataxia when intoxicated, and we used several tests of ambulatory coordination to assess this characteristic (table 6). The grid test (Belknap 1975) consists of a hardware cloth grid and measures stumbling errors per distance traveled as cases in which the mouse's foot falls through the mesh. FAST mice were found in one experiment to make more errors in this apparatus than SLOW mice (Phillips et al. 1989). A dose-response study suggested that this difference might be apparent only in a limited range of EtOH doses and that it was greater in mice from the second replicate. FAST and SLOW mice were also tested on a constant-speed rotarod after 2.5 g/kg of EtOH; FAST mice were more ataxic than SLOW mice on this task as well. When mice were tested on a static rod for latency to lose balance after 2.0 g/kg of EtOH, there were no differences between FAST and SLOW mice. These mice were again tested 30 min after injection on a larger rod that was accelerated at a constant rate. Latency to fall did not differ between the lines under these conditions. In summary, several measures assessing different aspects of locomotor incoordination yielded results suggestive of a genetic correlation between sensitivity to EtOH-induced ACT and EtOH-induced ataxia, but the results were not entirely consistent (table 6). Whether this is because the tasks assess different neural substrates or because the apparent genetic correlation is weak or spurious remains to be determined.

Generalization to Other Drugs

It was of interest to determine whether selection for sensitivity to EtOH-induced activation would lead to concomitant differential sensitivity to activation by other drugs. Table 7 presents abbreviated dose-response curves for the drugs discussed below. When FAST and SLOW mice of S_9 were tested for sensitivity to several doses of pentobarbital, only the replicate 1 lines differed. The source of this line difference appeared to be a large disparity in baseline activity, with a questionable difference in drug response. In a similar study with FAST and SLOW lines from both replicates from S_{11} given diazepam, a difference favoring greater activation in the FAST versus

Two Measures of Sensitivity

Table 6.—Summary of experiments of EtOH ataxia in FAST and SLOW mice

Test	Relative EtOH impairment
Grid test (1 dose)	FAST>SLOW
Grid test (dose response)	FAST>SLOW (at some doses)
Constant speed rotarod	FAST>SLOW
Dowel balancing	FAST=SLOW
Accelerating rotarod	FAST=SLOW

Table 7.—Differential generalization of activation to other drugs in FAST and SLOW mice

Drug	Dose (mg/kg)	Beam interruptions ^a			
		Replicate 1		Replicate 2	
		FAST	SLOW	FAST	SLOW
Pentobarbital	0	395±65	217±24	220±62	266±55
	10.00	264±23	247±20	236±35	246±46
	20.00	243±11	202±31	303±51	295±36
Diazepam	0	1,725±127	1,063±201	1,240±152	1,254±122
	2.50	1,818±217	1,476±170	1,334±161	1,207±134
	10.00	1,734±226	1,440±115	1,605±170	1,132±200
d-Amphetamine	0	1,390±57	1,417±177	1,212±79	1,168±77
	1.25	1,574±152	1,135±111	1,198±184	1,181±202
	5.00	1,813±294	1,755±200	1,461±171	1,013±190

^aMean ± SE beam interruptions for groups of eight mice during the 5-min period immediately after injection. A different set of monitors was used for the pentobarbital experiment than for the other drug tests, accounting for the large difference in activity counts between experiments.

SLOW mice of replicate 2 was found. The effect of diazepam in replicate 1 mice was more difficult to assess, again because of a large baseline activity difference between the two lines. S_{11} mice were also tested for d-amphetamine activation. In this study, FAST-2 mice were activated while SLOW-2 mice were not, but this difference did not appear in replicate 1 mice. Given the high variability in these studies and the fact that they are unreplicated, it is premature to draw conclusions about these drug responses and the genetic correlates of EtOH-stimulated activity.

Other Correlated Responses to Selection

In mice from S_7 - S_8 , the latency to lose and duration of loss of righting reflex (RR) were studied in FAST and SLOW mice injected with 4 g/kg of EtOH. BEC values were determined when RR was regained. FAST mice were more sensitive than SLOW mice to EtOH-induced loss of RR, as evidenced by their significantly lower BEC values at regaining RR (table 8). The lines did not differ either in latency to lose RR or in duration of loss of RR, but these measures include variables related to absorption, distribution, and elimination of EtOH as well as neurosensitivity (Phillips et al. 1989). FAST and SLOW mice did not differ in sensitivity in EtOH-induced HT, as indexed by the maximal change from baseline temperature either 30 to 60 min after injection of 3 g/kg of EtOH (Phillips et al. 1989) (figure 4). Although there may be pleiotropic effects of genes underlying EtOH activation on sensitivity to EtOH sedation, more experiments will be needed to verify this possibility.

Finally, we assessed the possibility that FAST and SLOW mice might differ in dopamine receptor sensitivity by administering different doses of apomorphine and testing for stereotypic climbing (Protais et al. 1976). Although apomorphine produced dose-dependent increases in climbing in all four selected lines, the lines did not differ in sensitivity.

Cryopreservation

In collaboration with Dr. Peter Mazur at the Oak Ridge National Laboratories, we have attempted to provide for the long-term maintenance of

Two Measures of Sensitivity

Table 8.—Neurosensitivity to EtOH-induced loss of RR in FAST and SLOW mice

Line	BEC at righting (mg/ml) ^a
FAST-1	4.10 ± 0.09
SLOW-1	4.33 ± 0.12
FAST-2	3.78 ± 0.08
SLOW-2	4.16 ± 0.09

^aMean ± SE for groups of 18-21 mice tested after administration of 4 g/kg of EtOH.

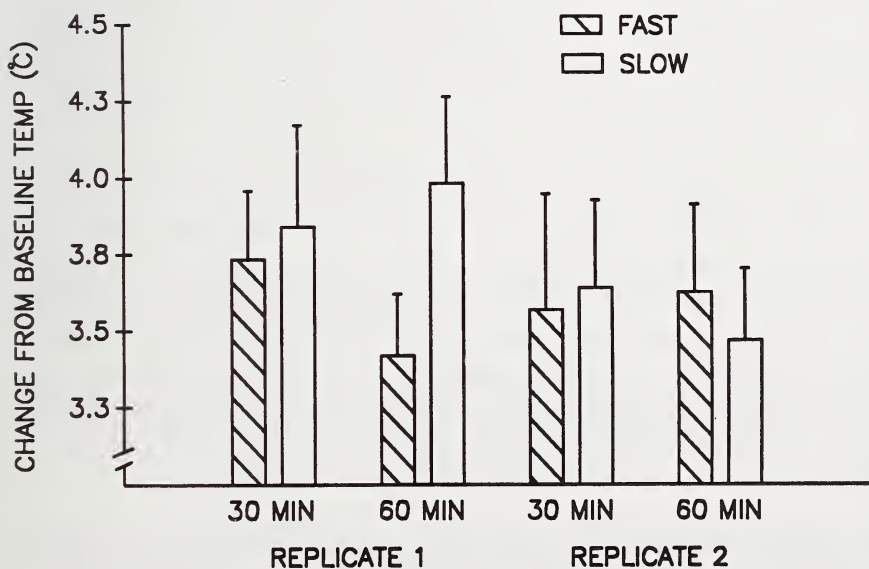


Figure 4.—Mean ± SE reduction from baseline body temperature after injection of 3 g/kg of EtOH in FAST and SLOW mice of both replicates.

the FAST/SLOW and HOT/COLD selected lines by cryopreserving embryos from the sixth selected generation of each line. A total of 200-300 embryos from each line, including CON lines, were frozen in Dr. Mazur's laboratory and are maintained at absolute zero to ensure against catastrophic loss of the genetic lines. Furthermore, we will repeat the cryopreservation in mice from S_{12} and future generations. This procedure will allow us to recapitulate the selection in cross-section at some later date in order to test any emergent hypotheses of correlated responses to selection. For example, if we find that FAST mice from S_{12} show a greater neurosensitivity to ethanol's effects on catecholamine turnover than do SLOW mice, we will be able to reconstitute mice of the genotype representing S_6 from cryopreserved embryos and see whether a tendency for the line difference was already apparent.

Summary

This paper has reviewed the progress of lines of mice genetically selected for sensitivity to EtOH-induced HT and to EtOH-induced locomotor activation in an open field. Selection for sensitivity and resistance to EtOH-induced HT has produced robust responses to selection in both replicate sets of lines. Selection has been asymmetrical: although it has been possible to select for increased HT, the HOT lines have not developed much smaller HT responses over generations. A number of correlated responses to selection have been documented in the HOT and COLD lines. This genetic difference generalizes to several other alcohols and depressants, suggesting that these compounds produce HT by the same mechanisms as does EtOH. Generalization is not complete, however: the HOT and COLD lines do not differ in sensitivity to a number of agents producing HT through presumably specific effects on neurotransmitter receptors. HOT mice, genetically insensitive to EtOH-induced HT, do not develop tolerance to this effect of EtOH.

FAST and SLOW lines have diverged less markedly during selection than have HOT and COLD mice. The response to selection has been asymmetrical in this selection as well. FAST lines show increasing activation over generations of selection, while SLOW lines show no decrease in

response. One SLOW line is in fact increasing in responsiveness despite selection against this character. Consistent with the lesser degree of divergence in the selected lines, the existence of correlated responses to selection is less clearly revealed in FAST and SLOW lines. Some evidence supports the development of a positive correlation between activation and ataxia. FAST mice are more sensitive than SLOW mice to EtOH anesthesia. They may be more sensitive than SLOW mice to amphetamine- and diazepam-induced activation. There is no consistent difference between the lines in sensitivity to EtOH-induced HT, or in sensitivity to apomorphine-induced stereotypy. The general results so far in both selections are encouraging, and we expect that future experiments will use these lines to address questions regarding the relevant mechanisms of action of EtOH.

References

- Belknap, J.K. The grid test: A measure of alcohol and barbiturate-induced impairment in mice. *Behav Res Methods Instrument* 7:66-67, 1975.
- Belknap, J.K.; Rabbe, J.C.; and Laursen, S.E. Ethanol and diazepam withdrawal convulsions are extensively co-selectively bred to be either sensitive or resistant to ethanol in WSP and WSR mice. *Life Sci* 44:2075-2080, 1989.
- Crabbe, J.C. Sensitivity to ethanol in inbred mice: Genotypic correlations among several behavioral responses. *Behav Neurosci* 97:280-289, 1983.
- Crabbe, J.C. Genetic differences in locomotor activation in mice. *Pharmacol Biochem Behav* 25:289-292, 1986.
- Crabbe, J.C.; Deutsch, C.M.; Tam, B.R.; and Young, E.R. Environmental variables differentially affect ethanol-stimulated activity in selectively bred mouse lines. *Psychopharmacology* 95:103-108, 1988a.
- Crabbe, J.C.; Feller, D.M.; and Dorow, J.S. Sensitivity and tolerance to ethanol-induced hypothermia in genetically selected mice. *J Pharmacol Exp Ther* 249:456-461, 1989.

Selective Breeding for Initial Sensitivity

- Crabbe, J.C.; Janowsky, J.; Young, E.; Kosobud, A.; Stack, J.; and Rigter, H. Tolerance to ethanol hypothermia in inbred mice: Genotypic correlations with behavioral responses. *Alcoholism Clin Exp Res* 6:446-458, 1982.
- Crabbe, J.C.; Kosobud, A.; Feller, D.J.; and Phillips, T.J. Using selectively bred mouse lines to study genetically correlates related to alcohol. In: Kuriyama, K.; Takada, A.; and Ishi, H. *Biomedical and Social Aspects of Alcohol and Alcoholism. Excerpta Med Int Congr Biomed Res Alcoholism*. Excerpta Medica International Congress Series 805. 1988b. pp. 427-430.
- Crabbe, J.C.; Kosobud, A.; Tam, B.R.; Young, E.R.; and Deutsch, C.M. Genetic selection of mouse lines sensitive (COLD) and resistant (HOT) to acute ethanol hypothermia. *Alcohol Drug Res* 7:163-174, 1987a.
- Crabbe, J.C.; Kosobud, A.; Young, E.; and Janowsky, J. Polygenic and single-gene determination of response to ethanol in BXD/Ty recombinant inbred mouse strains. *Neurobehav Toxicol Teratol* 5:181-187, 1983a.
- Crabbe, J.C.; Kosobud, A.; Young, E.R.; Tam, B.R.; and McSwigan, J.D. Bidirectional selection for susceptibility to ethanol withdrawal seizures in mus musculus. *Behav Genet* 15:521-536, 1985.
- Crabbe, J.C., and Weigel, R.M. Quantification of individual sensitivities to ethanol in selective breeding experiments: Difference scores versus regression residuals. *Alcoholism Clin Exp Res* 11:544-549, 1987.
- Crabbe, J.C.; Young, E.R.; Deutsch, C.M.; Tam, B.R.; and Kosobud, A. Mice genetically selected for differences in open-field activity after ethanol. *Pharmacol Biochem Behav* 27:577-581, 1987b.
- Crabbe, J.C.; Young, E.R.; and Kosobud, A. Genetic correlations with ethanol withdrawal severity. *Pharmacol Biochem Behav* 18(Suppl. 1):541-547, 1983b.
- Falconer, D.S. *Introduction to Quantitative Genetics*. 2d ed. New York: Longman, 1983.

Two Measures of Sensitivity

- Feller, D.J., and Crabbe, J.C. Effect of receptor-specific drugs in mice selected for differential sensitivity to the hypothermic action of ethanol. (Submitted a).
- Feller, D.J., and Crabbe, J.C. Effect of alcohols and other hypnotics in mice selected for differential sensitivity to the hypothermic action of ethanol. (Submitted b)
- Hill, W.G. Estimation of realized heritabilities from selection experiments. I. Divergent selection. *J Biometric* 28:747-765, 1972a.
- Hill, W.G. Estimation of realized heritabilities from selection experiments. II. Selection in one direction. *J Biometric* 28:767-780, 1972b.
- Horowitz, G.P., and Dudek, B.C. Behavioral pharmacogenetics. In: Fuller, J.L., and Simmel, E.C., eds. *Behavioral Genetics: Principles and Actions*. Hillsdale, NJ: Lawrence Erlbaum Associates, 1983. pp. 117-154.
- Kalant, H., and Le, A.-D. Effects of ethanol on thermoregulation. *Pharmacol Ther* 23:313-364, 1984.
- McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. (ADM)81-1133. Washington, D.C.: Supt. of Docs., U.S. Govt. Print. Off., 1981.
- Phillips, T.J.; Terdal, E.S.; and Crabbe, J.C. Response to selection for sensitivity to ethanol hypothermia and locomotor activation: genetic analyses. (Submitted.)
- Phillips, T.J.; Limm, M.; and Crabbe, J.C. Correlated behavioral responses as potential predictors of sensitivity to ethanol's rewarding effects and addiction liability. In: Kiianmaa, K.; Tabakoff, B.; and Saito, T. *Genetic Aspects of Alcoholism*. Finnish Foundation for Alcohol Studies, Painokaari, Helsinki. 37:197-206, 1989.
- Protais, P.; Costentin, J.; and Schwartz, J.C. Climbing behavior induced by apomorphine in mice: A simple test for the study of dopamine receptors in striatum. *Psychopharmacology* 50:1-6, 1976.

Reed, T.E. Three heritable responses to alcohol in a heterogeneous randomly mated mouse strain. *J Studies Alcohol* 38:618-632, 1977.

Discussion

COLLINS: None of the speakers this morning have discussed the issue of "who cares." In spite of the fact that we have had these selected lines for many years, it is only in the last few years that people have done studies on the ultimate animal model, i.e., the human, that address the question of whether or not a difference in sensitivity to alcohol is related to alcoholism in humans. Dr. Marc Schuckit's work indicates that there is a genetic component that influences many of the effects of ethanol in humans. Individuals at risk for development of alcoholism are less sensitive to several alcohol effects than are those at lesser risk for development of alcoholism. This gives added credence to the work that we are doing with animal models of acute sensitivity to ethanol, in addition to increasing the usefulness of animal genetics in unraveling the basic mechanisms of ethanol's action.

With respect to animal models and selective breeding, perhaps we should ask the question, "Is it so terrible when we have a failure?" When I look at the FAST and SLOW mice, I come to the conclusion that there is not a lot going on there. I do not see much evidence of selection occurring. This may be due to the complexity of the measure. If there is a lot of noise in the measure, it may be difficult to pull out the gene action from the noise or environmental effects. Perhaps the environmental influence is incredibly high and there is just not much detectable influence of genotype. To my mind, finding out which alcohol effects are not markedly influenced by genetic factors is just as important as finding out which effects are.

The other thing I want to mention before general discussion is the issue of correlated responses. It is unfortunate that we did not have the opportunity to measure correlated responses to sleep time as the SS and LS mice were being developed. The use of correlated responses allows the pharmacologist to answer such questions as "Does alcohol act by the same mechanism of action as does pentobarbital?"; "Does alcohol work by interaction with the GABA-benzodiazepine receptor?" The ability to measure these correlated responses as the animals are being separated is much better than measuring

the responses after selection is done or in 27 recombinant inbred strains or, as I have done, in F_1 and F_2 and the backcross generations.

SPUHLER: As a starting point, correlated responses can also be looked at in a series of inbred strains if other approaches are not available.

COLLINS: With regard to the use of inbred strains, one should remember that a mouse is a mouse is still a mouse. Something like 95 percent of all genes in mice are the same so that we have relatively little to play with. The second problem is that we have relatively few inbred strains really, and many of these are related in their ancestry. So while one can do inbred strain comparisons, if the behavioral trait you are interested in is tightly linked to the biochemical trait, one can achieve a very high correlation between the two but it may be entirely fortuitous. The process of selection is one whereby linkages are broken up and a closer approach to cause-and-effect correlations can be made. This is why, when Dr. Erickson said that inbreeding should start early in the selective breeding programs, a number of us were shaking our heads, because we want to maintain the diversity in all the genes that are not related to the trait for which we are selecting.

DEITRICH: I would like to ask Dr. Crabbe about his situation where one line is positive in some measure but the replicate line is negative. In my system of classification, I would call that a false-positive.

CRABBE: I guess that depends on whether you're an optimist or a pessimist. In the case of the FAST/SLOW selection, we see a highly significant difference between the lines in each replicate of the experiment, but the difference is much larger in one replicate than in the other. This appears to be because one of the four lines, the SLOW-1 line, is responding unexpectedly. This line is still showing increasing activation across generations, although we are breeding them for decreased activation. The other SLOW line continues to decrease as expected, and both FAST lines continue to increase. We tend to look at this as a statistical question of estimating the true main effect of genotype from a two-way ANOVA design with factors line and replicate. When we apply such an analysis, we see a significant main effect of line, which says to us that there is indeed a genetic basis for the differences between FAST and SLOW mice. The significant line-by-replicate interaction supports the obvious fact that this difference is

bigger in one replicate. Our view is that we can't tell at this point whether the large difference between FAST-2 and SLOW-2 mice is a "false-positive," to use Dr. Deitrich's term, or whether the smaller difference between FAST-1 and SLOW-1 mice is a "false-negative." One way to resolve the issue of which replicate of the FAST/SLOW selection is the more representative of the true genetic situation would be to repeat the selection in several more pairs of lines. You can't tell which is the true response with only two experiments.

DEITRICH: That was the point made earlier, that you can't tell at this point if these are, in fact, false-positives, but for immediate purposes that is what we have called them. Later results or better ways of measuring the responses may show that some of these differences are in fact causally related to the selected genotype.

CRISWELL: Perhaps this is premature, but it seems to me that we have a lot of data already available and perhaps we could sit down and list all that we know about ethanol's action from these studies and come up with a pretty good idea of what ethanol is doing. Since we can selectively breed for one effect of alcohol and not another, it would appear that alcohol has specific effects.

COLLINS: In response to what you said, I scribbled a note to myself that quotes Gerry McClearn a number of years ago when he said, "You get what you select for." It is clear that the models don't always agree. For example, the LS and SS mice and the HAS and LAS rats do not agree with respect to the corticosterone release story. One aspect that may not be apparent to those who do not work with these animals is that there is a tremendous effect of the concentration of alcohol that is slammed into the peritoneal cavity. When a graduate student of mine got this result, I began to think how the SS and LS mice were selected. They were selected on the basis of a 30 percent ethanol solution given IP. It turns out the LS mice are very sensitive to concentration, while the SS mice are not. The fact that the corticosterone response in HAS and LAS rats' response to ethanol is not seen may be due to the fact that with the rats 15 percent ethanol was given while in the mice 30 percent was given. Another example is in the selection for withdrawal sensitivity selections going on at Colorado and in Oregon. We select for withdrawal sensitivity after giving the ethanol orally, while Dr. Crabbe gives

the ethanol through the air. Differences in animal models may be due to differences in how we give the drug.

Cell Membranes: Structure and Function

Cell Membrane Fluidity as a Determinant of Intoxication: Status of the Hypothesis

*Dora B. Goldstein*¹

The hypothesis that disordering of membrane lipid bilayers directly or indirectly causes intoxication (and thereby leads to tolerance and physical dependence) has been tested fairly extensively over several years by my laboratory and others. It is time for an evaluation and a vigorous attempt to disprove the hypothesis.

Qualitative Correlations Between Disorder and Intoxication

Behavioral effects of alcohol-like drugs correlate with lipid solubility. The disorder hypothesis began with the reports of H. H. Meyer, E. Overton, and K. H. Meyer (Meyer and Hemmi 1935) that ethanol is a weak anesthetic with a potency that matches its low oil-water partition coefficient. Ethanol fits among many other anesthetic drugs whose potencies are related to their membrane-buffer partition coefficients (Seeman 1972). A more recent observation is that the behavioral potency of ethanol matches its ability to disorder plasma membranes *in vitro*, as assessed by electron paramagnetic resonance (EPR) or fluorescence polarization techniques. The presence of ethanol in the membrane causes both disorder and intoxication. The

¹Department of Pharmacology, Stanford University, School of Medicine, Stanford, CA 94305.

hypothesis we are examining here is that structural disordering of membranes *causes* intoxication.

Certainly membrane disordering *in vitro* correlates with behavioral intoxication. Examples are presented below.

Pharmacologic Correlations

Many studies, of which that of McCreery and Hunt (1978) was the most extensive, have shown that alcohols and related central nervous system (CNS) depressant drugs have behavioral potencies that reflect their membrane-buffer partition coefficients. The ability of alcohols to disorder spin-labeled synaptosomal membranes also matches their partition coefficients and thus their hypnotic potencies (Lyon et al. 1981).

Some quite lipid-soluble compounds, including long-chain alkanols and alkanes, do not cause CNS depression (McCreery and Hunt 1978; Pringle et al. 1981). This observation is irrelevant to the disorder hypothesis because these compounds in general may not disorder membranes and may be too insoluble in water ever to reach the brain *in vivo*.

Temporal Correlations

After chronic exposure of animals to ethanol, their membranes become relatively insensitive to the disordering effect of ethanol *in vitro*, just as the animals themselves become behaviorally tolerant. This "membrane tolerance" is elicited by any of the common methods of chronic administration of ethanol and appears in erythrocyte membranes of mice (Chin and Goldstein 1977) and alcoholic humans (Beaugé et al. 1985), brain microsomes (Aloia et al. 1985), hepatic microsomes (Ponnappa et al. 1982; Taraschi et al. 1986) and mitochondria (Rottenberg et al. 1981), and plasma membranes of hepatocytes (Polokoff et al. 1985) and brain (Chin and Goldstein 1977; Lyon et al. 1981; Rottenberg et al. 1981; Harris et al. 1984; Mullin et al. 1987; Leguicher et al. 1987). In liver microsomes from alcohol-treated rats, the membrane lipids alone, extracted and protein free, are resistant to ethanol (Taraschi et al. 1986), but in brain synaptosomes, tolerance is not a property of the extracted lipids (Harris et al. 1984).

Decreased partition coefficients for ethanol would be a sufficient explanation for tolerance. Measurement of partition coefficients for ethanol is extremely difficult because ethanol remains mostly in water in any aqueous suspension of membranes. A high proportion of the ethanol is in the trapped water within a membrane pellet; corrections using tritiated water are required but are difficult to carry out with precision. Nevertheless, Rottenberg et al. (1981) observed decreased partitioning of ethanol and other lipid-soluble compounds in the mitochondrial and synaptosomal membranes of ethanol-tolerant rats. More recently, Leguicher et al. (1987) reported similar results and showed that the sensitivity of rat synaptosomal membranes to ethanol-induced disordering varies with the partition coefficients.

E. Rubin's group has reported that the observed membrane tolerance in rat liver microsomes results from a change in only one class of phospholipid, the phosphatidylinositol (PI) (Taraschi et al. 1986). When the extracted phospholipids were separated and reconstituted, a small amount of PI from the alcohol-treated rats sufficed to make normal membranes resistant to added ethanol. Because PI is a minor component of membranes, it does not seem likely that disordering of PI alone was responsible for all the observed change-in-order parameter on addition of ethanol to normal membranes. Therefore, the substitution of PI from alcohol-treated animals must have actually prevented the disordering of the major components of the membranes, such as phosphatidylcholines and phosphatidylethanolamines.

Genetic Correlations

Short-sleep (SS) mice as well as ethanol-resistant individual mice of a genetically heterogeneous stock (HS) have brain and erythrocyte membranes that are less sensitive to ethanol-induced disordering than are membranes of mice that are behaviorally sensitive to ethanol (Goldstein et al. 1982). The genetic difference seems to be located near the surface of the membrane, as it is seen with surface probes but not deep ones in both EPR (Goldstein et al. 1982; Perlman and Goldstein 1984) and fluorescence polarization (Harris et al. 1988) experiments. Harris et al. (1988) also reported that the line difference in sensitivity of membrane order was not seen with longer chain alcohols that penetrate deeper into the bilayer than ethanol does. This recalls the reports that there is no line difference in the hypnotic or hypothermic effects of longer chain alcohols (Howerton et al. 1983) or in the

hypnotic effect of halothane, a highly lipid soluble anesthetic (Baker et al. 1980). Harris et al. (1988) observed no line difference in sensitivity to disordering in lipid extracts of the membranes and reported that the line difference in magnitude of disordering was abolished in the presence of calcium. Thus, it appears that the membranes of LS and SS mice differ in some surface component.

Physical Correlations

As with ethanol, an increase in temperature disorders membranes *in vitro*. An increase in body temperature in intact animals increases the lethality of ethanol (Dinh and Gailis 1979; Malcolm and Alkana 1982), which suggests that the hypothermia evoked by acute ethanol intake is protective, offsetting the disorder in membranes. Ethanol-treated goldfish seek a cooler environment than controls do (O'Connor et al. 1988). In cultured cells, ethanol and heat evoke a similar reaction that includes thermotolerance, tolerance to lethal effects of ethanol (Li and Hahn 1978), and often the production of heat shock proteins (Li 1983). Production of heat shock proteins is a rather nonspecific reaction to "stress" that may or may not be informative in this connection.

Another physical interaction is that increased hydrostatic pressure, which may force membranes into an ordered configuration, counteracts the effects of anesthetics (Miller et al. 1973), including ethanol (Alkana and Malcolm 1980). Miller's "critical volume hypothesis" postulates that anesthesia occurs when the anesthetic has expanded a membrane by a critical amount; pressure reverses the effect. The pressures necessary to reverse anesthesia are of the order of 100 atm, but the data of Alkana and Malcolm (1980) indicate that a much lower pressure, 4-8 atm, suffices for an appreciable shortening of ethanol-induced hypnosis.

These correlations are strong and coherent, but they do not prove causation. Can they all be explained by differences in partition coefficients? Certainly the lipid solubility data do not tell us much except that the anesthetic drugs can partition into hydrophobic sites. The tolerance and genetic data pertain to differences among membranes rather than drugs; they indicate that some membranes are more sensitive than others, either intrinsically or because the drugs partition easily into them. The evidence that LS/SS line differences

seem to be localized near the aqueous interface suggests that differences in partition coefficient do not mediate the line difference in sensitivity to hypnosis. Partition coefficients could not explain differential genetic effects at different membrane domains.

There are exceptions to the relationships between disordering and intoxication, as discussed below.

Development and Aging

In young, developing animals and also in aged animals, there are changes in behavioral sensitivity to ethanol without equivalent changes in sensitivity of membranes to disordering. Very young rats are quite resistant to ethanol (Hollstedt et al. 1980). They lose the resistance quickly, but this change is not accompanied by increased sensitivity of membranes to disordering by ethanol *in vitro* (Hitzemann and Harris 1984). Similarly, mice become quite sensitive to ethanol as they age, whereas their membranes become less so (Armbrecht et al. 1983). Aging is a complex process, including so many components that any one aspect (such as membrane order) may be difficult to identify.

Stimulation of Adenylate cyclase

Alcohols disorder membranes and stimulate adenylate cyclase. Butanol is more potent than ethanol in both respects, but at equivalent degrees of disordering, ethanol is more potent than butanol in stimulating the enzyme (Rabin et al. 1986). Because we do not know the relationship of cyclase action to intoxication, this information does not quite address the question I am dealing with here. But further pieces of straightforward evidence like this, pertaining to a behavioral effect, would go a long way toward resolving the question.

Quantitative Relationships Between Ethanol and Temperature

The fact that ethanol and warming have similar disordering effects on membranes offers the best opportunity to test the disorder hypothesis. Numerical measures of disorder in biomembranes are obtained from EPR

spectra of spin-labeled membranes (to which a paramagnetic reporter molecule such as a doxylstearic acid has been added) or from the polarization (anisotropy) of fluorescence emitted by a dye that is incorporated into the bilayer. Mobility of the signal molecule presumably reflects that of the membrane components that surround it, i.e., the overall mobility of the bulk lipids. The magnitude of disordering can be measured as the percent decrease in the EPR order parameter or the fluorescence anisotropy. Comparing ethanol-induced and temperature-induced disordering, we find that warming by less than 1 degree disorders membranes more strongly than does a near-lethal concentration of ethanol (table 1). Obviously such small temperature changes, which occur daily in the course of mammalian circadian rhythm, do not cause behavior resembling severe intoxication.

The insensitivity of the membrane order parameter or fluorescence anisotropy to ethanol (relative to temperature) indicates that intoxication cannot be mediated by disordering of the bulk lipid. But biomembranes are not homogeneous. Perhaps there are more sensitive domains such as the interface of lipid and protein or the very center of the membrane between the monolayers or at the edges of gel domains in a membrane that includes patches of gel-phase lipid in a matrix of fluid-phase lipid (Trudell 1977). It is still reasonable to postulate that ethanol may work by disordering some specific sensitive region where it has large effects relative to warming. To disprove the disorder hypothesis, we must rule out any such sites.

How much increase in potency is needed at the hypothetical sensitive site? We can estimate this factor by calculating (at each site) the change in temperature that would be required to produce a decrease in order equivalent to that produced by 100 mM ethanol. We need to find a site where 100 mM ethanol, a strongly intoxicating, often lethal concentration *in vivo*, is equivalent to several degrees of heating. For comparison, consider the EPR data with 5-doxylstearic acid as spin label in mouse synaptosomal plasma membranes. This probe reports from a relatively stable region of the bilayer, where neither ethanol nor temperature has a strong effect. At 19 °C, a temperature at which it is feasible to examine membranes with several different spin labels, 100 mM ethanol decreases the order parameter by 0.18 percent, whereas increasing the temperature decreases the order parameter 1 percent per degree. The response to temperature does not change much over the range from 10 to 28 °C, but the response to ethanol is larger at the

Table 1.—Comparison of the effects of raising the experimental temperature with the effects of adding ethanol^a

Probe	Temperature (°C)	Order ^b	Change in order		Degrees/ 100mM
			percent/ degree	percent/ 100mM	
5-DS	10.5	0.770		-0.09	0.09
	19.0	0.707	-1.0	-0.18	0.18
	28.0	0.643	-1.0	-0.28	0.28
12-DS	10.5	0.601		-0.34	0.20
	19.0	0.517	-1.7	-0.67	0.40
	28.0	0.442	-1.7	-0.98	0.58
DPH	26.0	0.260		-0.73	0.46
	37.0	0.221	-1.6	-1.02	0.64

^aIn EPR and fluorescence polarization experiments with mouse synaptosomal plasma membranes, the order parameter was measured with the spin labels 5-doxylstearic acid and 12-doxylstearic acid (5-DS and 12-DS, respectively), and the fluorescence anisotropy was measured with DPH. The percent change in these measures per degree of warming or per 100 mM added ethanol was calculated, and the ratio of these two values indicating how much rise in temperature can be mimicked by 100 mM ethanol, is shown in the rightmost column. Data are from Chin and Goldstein (1981) and D.B. Goldstein (unpublished data).

^bValues are order parameters for the doxylstearic acid probes or fluorescence anisotropy for DPH at the experimental temperatures shown in the next column.

higher temperatures. Ethanol at 100 mM is thus equivalent to 0.1 degree at 10.5 °C, 0.2 degree at 19 °C, and 0.3 degree at 28 °C.

Deeper in the membrane, we can examine a more ethanol sensitive region with the 12-doxylstearic acid spin label. Here at 19 °C the order parameter is lower than it is near the membrane surface, and 100 mM ethanol disorders by 0.67 percent. But this region is also more sensitive to temperature. Increasing the temperature from 10.5 to 28 °C decreases the order parameter uniformly by 1.7 percent per degree. Again, ethanol disorders more strongly at the higher temperatures; 100 mM ethanol is equivalent to about 0.2-0.6 degrees of warming over this temperature range (table 1). This is not enough, but it is different from what we saw at the surface and suggests that we might be able to find a still more sensitive site. Fluorescence anisotropy is measured with a probe (diphenylhexatriene [DPH]) that is distributed throughout the membrane. When membrane order is measured with this probe, 100 mM ethanol is equivalent to 0.6 degree at most, as with 12-doxylstearic acid (table 1). Among the most ethanol-sensitive membranes we have observed are cholesterol-free liposomes of egg lecithin, spin labeled with 12-doxylstearic acid and run at 15 °C (Chin and Goldstein 1981). Here 100 mM ethanol decreased the order parameter by a relatively large amount, 1.7 percent, but these bilayers were also quite sensitive to a rise in temperature. One degree of warming reduced the order parameter by 3.8 percent. Thus, 100 mM ethanol was equivalent to only 0.45 degree.

Liver microsomes, perhaps because they contain very little cholesterol, are much more sensitive to disordering than are the plasma membranes shown in table 1. They are disordered as much as 8 percent by 100 mM ethanol in EPR experiments with 12-doxylstearic acid (Taraschi et al. 1986). The response of liver microsomes to warming in the physiologic temperature range seems not very different from that of plasma membranes (Ponnappa et al. 1982). It does seem possible that intracellular membranes are a site of relevant disordering by ethanol.

Thus, by exploring the available regions of membranes, we have revealed some domains that are relatively sensitive to ethanol. Most of these regions are also sensitive to warming and in plasma membranes ethanol can only cause a disordering equivalent to less than 1 degree. Hepatic microsomes

are unlikely mediators of intoxication, but there may be small components of brain membranes that react like hepatic microsomes and may provide the sought-for sensitivity.

It is possible to estimate indirectly how much the partition coefficient of ethanol varies in its different sites of action, assuming that different behavioral responses to ethanol, or responses in different biologic systems, may be mediated at different kinds of membranes. By compiling data from the literature of the effects of homologous series of alcohols, we can estimate the factor by which the potency for a specific action increases with each additional carbon atom in the chain. This number reflects the partition coefficient for a methylene group into the particular membrane where ethanol works to produce that effect. I found that the factor varies somewhat, over a range from about 2 to about 4 (Goldstein 1989), giving some indication of a set of physically different sites of action in various species and organs.

Cell Membranes in Animals Chronically Exposed to Ethanol

Membrane tolerance, a consistent correlate of behavioral tolerance after chronic administration of ethanol, was discussed above. What about physical dependence, a condition that often accompanies tolerance? We may imagine that dependence would be represented by an increased intrinsic order in membranes of ethanol-treated animals, a change in the opposite direction from the acute effect of ethanol and therefore perhaps representing a withdrawal effect. Such changes often do occur but not so uniformly as membrane tolerance. Tolerance can be observed under conditions in which the intrinsic membrane order is normal. The increased order in ethanol-treated animals, when it does appear, is intriguing because it suggests an adaptive response to the initial disordering. The disorder seems to have been perceived by some physiologic mechanism and judged to need correcting. It does not necessarily follow that the disruption in need of correcting was related to intoxication.

We observed increased order in brain membranes of ethanol-treated mice when used 12-doxylstearic acid as spin label but not with the near-surface

probe 5-doxylstearic acid (Lyon and Goldstein 1983). The 12-doxy results have been confirmed with fluorescence polarization, again using a deep probe (Perlman and Goldstein 1983; Harris et al. 1984). Attempts to find the chemical reason for the changed physical properties of membranes have been disappointing, just as they have been for tolerance.

In striking contrast to the plasma membranes of brain and erythrocytes and even to hepatic intracellular membranes, liver plasma membranes become more fluid after chronic treatment with ethanol, either in intact animals (Yamada and Lieber 1984; Kim et al. 1988) or in cultured hepatocytes (Polokoff et al. 1985). The effect is ascribed to alcohol metabolism (Polokoff et al. 1985) and to abnormal amounts of cholesterol esters and retinoids in the hepatic plasma membrane (Kim et al. 1988). Clearly, the organ-specific metabolic activity of the liver affects the response of its plasma membranes to chronic alcohol exposure.

Because an increased membrane content of cholesterol can order membranes and reduce their sensitivity to ethanol (Chin and Goldstein 1981, 1984), we looked to see whether ethanol treatment might increase the rate of transfer of cholesterol into membranes. Indeed, ethanol *in vitro* did accelerate the equilibration of red cell membranes with external cholesterol, an effect resembling that of an increase in temperature (Daniels and Goldstein 1982). Furthermore, ethanol accelerated the transfer into membranes of palmitate relative to that of oleate, a more flexible and disordered fatty acid (Chin and Goldstein 1985). Either of these changes, if they occurred *in vivo*, might cause membrane tolerance and dependence. However, in a direct test of this idea, we treated Japanese quail with dietary cholesterol, greatly raising their serum cholesterol levels, and found that their brain membrane cholesterol levels were unaffected. Even adding ethanol to the high-cholesterol diet did not affect the well-regulated cholesterol levels of brain synaptosomal membranes (Chin and Goldstein 1984, unpublished data).

Whatever causes the hyperexcitable withdrawal state in whole animals may indeed be a property of membrane lipids, but if so it exists in places we cannot see. These could be the same hypersensitive regions we were looking for in attempts to explain the acute effects of ethanol relative to temperature. But in trying to explain chronic effects, we step too far from the data. We

take one doubtful step from observable bulk lipid to postulated sensitive regions where ethanol-specific effects occur and another giant step into the dark, complex area of responses to chronic drug administration. We can go no further without more data.

Summary

For decades we have known that the membrane-buffer partition coefficients of alcohols match their potencies as anesthetics. More recently, we have learned that membrane disordering also matches behavioral effects of alcohols. The membranes of ethanol-tolerant animals and genetically ethanol resistant mice are relatively resistant to ethanol-induced disordering. Ethanol and increased temperature have additive behavioral and disordering effects. These data suggest that disorder may cause intoxication. Quantitatively, however, the disorder hypothesis holds up only if there exists a site of action of ethanol where disorder is much more strongly increased by ethanol, relative to temperature, than present data show. Chronic administration of ethanol causes some membranes to become more ordered and liver plasma membranes to become less so. There is no clear relationship between intrinsic membrane order and physical dependence. Clearly, ethanol works at a hydrophobic site but not by disordering the bulk lipid.

References

- Alkana, R.L., and Malcolm, R.D. Antagonism of ethanol narcosis in mice by hyperbaric pressures of 4-8 atmospheres. *Alcoholism* 4:350-353, 1980.
- Aloia, R.C.; Paxton, J.; Daviau, J.S.; van Gelb, O.; Mlekusch, W.; Truppe, W.; Meyer, J.A.; and Brauer, F.S. Effect of chronic alcohol consumption on rat brain microsome lipid composition, membrane fluidity and $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity. *Life Sci* 36:1003-1017, 1985.
- Armbrecht, H.J.; Wood, W.G.; Wise, R.W.; Walsh, J.B.; Thomas, B.N.; and Strong, R. Ethanol-induced disordering of membranes from different age groups of C57BL/6NNIA mice. *J Pharmacol Exp Ther* 226:387-391, 1983.

- Baker, R.; Melchior, C.; and Deitrich, R. The effect of halothane on mice selectively bred for differential sensitivity to alcohol. *Pharmacol Biochem Behav* 12:691-695, 1980.
- Beaugé, F.; Stibler, H.; and Borg, S. Abnormal fluidity and surface carbohydrate content of the erythrocyte membranes of alcoholic patients. *Alcoholism* 9:322-326, 1985.
- Chin, J.H., and Goldstein, D.B. Drug tolerance in biomembranes: A spin label study of the effects of ethanol. *Science* 196:684-685, 1977.
- Chin, J.H., and Goldstein, D.B. Membrane-disordering action of ethanol. Variation with membrane cholesterol content and depth of the spin label probe. *Mol Pharmacol* 19:425-431, 1981.
- Chin, J.H., and Goldstein, D.B. Cholesterol blocks the disordering effect of ethanol in biomembranes. *Lipids* 19:929-935, 1984.
- Chin, J.H., and Goldstein, D.B. Differential effects of ethanol on uptake of saturated and unsaturated fatty acids into red cell membranes *in vitro*. *Fed Proc* 44:1239, 1985.
- Daniels, C.K., and Goldstein, D.B. Movement of free cholesterol from lipoproteins or lipid vesicles into erythrocytes: Acceleration by ethanol *in vitro*. *Mol Pharmacol* 21:694-700, 1982.
- Dinh, T.K.H., and Gailis, L. Effect of body temperature on acute ethanol toxicity. *Life Sci* 25:547-552, 1979.
- Goldstein, D.B. Alcohol and biological membranes. In: Goedde, H.W., and Agarwal, D.P. eds. *Alcoholism: Biomedical and Genetic Aspects*. New York: Pergamon Press, 1988, pp. 87-98.
- Goldstein, D.B.; Chin, J.H.; and Lyon, R.C. Ethanol disordering of spin-labeled mouse brain membranes: Correlation with genetically determined ethanol sensitivity of mice. *Proc Natl Acad Sci USA* 79:4231-4233, 1982.

- Harris, R.A.; Baxter, D.M.; Mitchell, M.A.; and Hitzemann, R.J. Physical properties and lipid composition of brain membranes from ethanol tolerant-dependent mice. *Mol Pharmacol* 25:401-409, 1984.
- Harris, R.A.; Zaccaro, L.M.; McQuilkin, S.; and McClard, A. Effects of ethanol and calcium on lipid order of membranes from mice selected for genetic differences in ethanol intoxication. *Alcohol* 5:251-257, 1988.
- Hitzeman, R.J., and Harris, R.A. Development changes in synaptic membrane fluidity: A comparison of 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH). *Dev Brain Res* 14:113-120, 1984.
- Hollstedt, C.; Olsson, O.; and Rydberg, U. Effects of ethanol on the developing rat. II. Coordination as measured by the tilting-plane test. *Med Biol* 58:164-168, 1980.
- Howerton, T.C.; O'Connor, M.F.; and Collins, A.C. Differential effects of long-chain alcohols in long- and short-sleep mice. *Psychopharmacology* 79:313-317, 1983.
- Kim, C.-I.; Leo, M.A.; Lowe, N.; and Lieber, C.S. Effects of vitamin A and ethanol on liver plasma membrane fluidity. *Hepatology* 8:735-741, 1988.
- Leguicher, A.; Beaugé, F.; and Nordmann, R. Concomitant changes of ethanol partitioning and disordering capacities in rat synaptic membranes. *Biochem Pharmacol* 36:2045-2048, 1987.
- Li, G.C. Induction of thermotolerance and enhanced heat shock protein synthesis in Chinese hamster fibroblasts by sodium arsenite and by ethanol. *J Cell Physiol* 115:116-122, 1983.
- Li, G.C., and Hahn, G.M. Ethanol-induced tolerance to heat and to adriamycin. *Nature (London)* 274:699-701, 1978.
- Lyon, R.C., and Goldstein, D.B. Changes in synaptic membrane order associated with chronic ethanol treatment in mice. *Mol Pharmacol* 23:86-91, 1983.

- Lyon, R.C.; McComb, J.A.; Schreurs, J.; and Goldstein, D.B. A relationship between alcohol intoxication and the disordering of brain membranes by a series of short-chain alcohols. *J Pharmacol Exp Ther* 218:669-675, 1981.
- Malcolm, R.D., and Alkana, R.L. Temperature dependence of ethanol lethality in mice. *J Pharm Pharmacol* 35:306-311, 1982.
- McCreery, M.J., and Hunt, W.A. Physico-chemical correlates of alcohol intoxication. *Neuropharmacology* 17:451-461, 1978.
- Meyer, K.H., and Hemmi, H. Beiträge zur Theorie der Narkose. III. *Biochem Z* 277:39-71, 1935.
- Miller, K.W.; Paton, W.D.M.; Smith, R.A.; and Smith, E.B. The pressure reversal of general anesthesia and the critical volume hypothesis. *Mol Pharmacol* 9:131-143, 1973.
- Mullin, M.J.; Dalton, T.K.; Hunt, W.A.; Harris, R.A.; and Majchrowicz, E. Actions of ethanol on voltage-sensitive sodium channels: Effects of acute and chronic ethanol treatment. *J Pharmacol Exp Ther* 242:541-547, 1987.
- O'Connor, C.S.; Crawshaw, L.I.; Bedichek, R.C.; and Crabbe, J.C. The effect of ethanol on temperature selection in the goldfish, *Carassius auratus*. *Pharmacol Biochem Behav* 29:243-248, 1988.
- Perlman, B.J., and Goldstein, D.B. Ethanol and sodium valproate disordering of membranes from chronic ethanol treated mice. *Fed Proc* 42:2123, 1983.
- Perlman, B.J., and Goldstein, D.B. Genetic influences on the central nervous system depressant and membrane-disordering actions of ethanol and sodium valproate. *Mol Pharmacol* 26:547- 552, 1984.
- Polokoff, M.A.; Simon, T.J.; Harris, R.A.; Simon, F.R.; and Iwahashi, M. Chronic ethanol increases liver plasma membrane fluidity. *Biochemistry* 24:3114-3120, 1985.
- Ponnappa, B.C.; Waring, A.J.; Hoek, J.B.; Rottenberg, H.; and Rubin, E. Chronic ethanol ingestion increases calcium uptake and resistance to

- molecular disordering by ethanol in liver microsomes. *J Biol Chem* 257:19141-19146, 1982.
- Pringle, M.J.; Brown, K.B.; and Miller, K.W. Can the lipid theories of anesthesia account for the cutoff in anesthetic potency in homologous series of alcohols? *Mol Pharmacol* 19:4-55, 1981.
- Rabin, R.A.; Bode, D.C.; and Molinoff, P.B. Relationship between ethanol-induced alterations in fluorescence anisotropy and adenylate cyclase activity. *Biochem Pharmacol* 35:2331-2335, 1986.
- Rottenberg, H.; Waring, A.; and Rubin, E. Tolerance and cross-tolerance in chronic alcoholics: Reduced membrane binding of ethanol and other drugs. *Science* 213:583-585, 1981.
- Seeman, P. The membrane actions of anesthetics and tranquilizers. *Pharmacol Rev* 24:583-655, 1972.
- Taraschi, T.F.; Ellingson, J.S.; Wu, A.; Zimmerman, R.; and Rubin, E. Phosphatidylinositol from ethanol-fed rats confers membrane tolerance to ethanol. *Proc Natl Acad Sci USA* 83:9398-9402, 1986.
- Trudell, J.R. A unitary theory of anesthesia based on lateral phase separations in nerve membranes. *Anesthesiology* 46:5-10, 1977.
- Yamada, S., and Lieber, C.S. Decrease in microviscosity and cholesterol content of rat liver plasma membranes after chronic ethanol feeding. *J Clin Invest* 74:2285-2289, 1984.

Discussion: Cell Membrane Fluidity as a Determinant of Intoxication: Status of the Hypothesis¹

Ronald L. Alkana²

Introduction

From a strict pharmacologic perspective (Goodman et al. 1985), it is important to differentiate a drug's mechanism of action from its site of action and from the subsequent effects on the cascade. From this perspective, most of the presentations at this conference can be divided into three related topics (table 1): (1) possible mechanisms of action of ethanol; (2) possible sites of action of ethanol; and (3) genetic selection for differential sensitivity to the initial effects of ethanol. Given this framework, Dr. Goldstein's presentation of ethanol-induced changes in cell membrane fluidity (this

¹This research was supported in part by research grants RO1 AA03972 and AA05234 from the National Institute of Alcohol Abuse and Alcoholism.

²Alcohol and Brain Research Laboratory, School of Pharmacy, University of Southern California, Los Angeles, CA 90033.

Table 1.—Possible mechanisms and sites of action of ethanol

Mechanism of action (nonstereospecific)

- Perturbation
- Protein pocket
- Other

Sites of action (hydrophobic microenvironment)

General

- Membrane
- Lipid-protein interaction
- Protein
- Other

Specific

- GABA-benzodiazepine receptor chloride ionophore complex
- Ion channels (chloride, sodium)
- Coupling proteins
- Neuropeptides (opiates, corticotropin-releasing factor, neurotensin)
- Prostaglandin related
- Catecholamine related
- Acetylcholine related
- Other

Genetic selection for effects of ethanol

- SS and LS mice
- HOT and COLD mice
- FAST and SLOW mice
- AT and ANT rats
- HAS and LAS rats
- Other

volume) deals primarily with two of these topics: a possible mechanism and site of action for ethanol.

Membrane Fluidization Hypothesis

Dr. Goldstein focused her attention on the current status of the membrane fluidity hypothesis of the action of ethanol. This hypothesis suggests that ethanol-induced increases in cell membrane lipid fluidity represent the initial action leading to the intoxicating effects of ethanol. The following material briefly summarizes the evidence for and against the membrane lipid hypothesis presented by Dr. Goldstein, identifies key questions suggested by Dr. Goldstein's talk, and describes work, primarily from my laboratory, that addresses some of these questions.

Evidence Supporting the Membrane Fluidization Hypothesis

Dr. Goldstein reviewed the original evidence from Meyer and Overton linking the behavioral effects of general anesthetic drugs to their lipid solubilities (oil-water partition coefficients). She presented additional pharmacologic, genetic, physical, and temporal evidence supporting the membrane fluidity hypothesis. Specifically, she pointed out that intoxication potency correlates with lipid solubility and in vitro measurement of membrane disorder for anesthetic drugs. Genetic selection studies also support the hypothesis, since membranes obtained from animals inherently sensitive to the behavioral effects of ethanol (LS mice) are more sensitive to ethanol-induced fluidization than are membranes from lines selected for resistance to the behavioral effects of ethanol (SS mice). Studies showing that physical manipulation of body temperature and atmospheric pressure affect behavioral sensitivity to ethanol in the same way that they affect ethanol-induced fluidity changes also support the hypothesis. Finally, she discussed evidence from animals chronically exposed to ethanol. These studies indicate that brain cell membranes in animals exposed chronically to ethanol become resistant to the fluidizing effects of ethanol. This resistance could represent an adaptive response to the fluidizing effects of ethanol and could underlie

the development of tolerance to the effects of ethanol and physical dependence.

Collectively, this evidence points to a hydrophobic site of action for ethanol. But the identity (chemical nature and function) of the site or sites of action is unknown. On the basis of evidence that the fluidizing potency of alcohols varies with the type of membrane investigated, Dr. Goldstein suggested that there may be different sites of action for ethanol within the membrane. She also emphasized the importance of linking mechanistic studies to the target (site of action) and the effect of changes in function at the site on behavior.

Evidence Against the Membrane Fluidization Hypothesis

Two lines of evidence suggested by Dr. Goldstein represent major problems for the membrane fluidization hypothesis. First, recent work from Adron Harris' laboratory suggests that a potent membrane-fluidizing agent does not cause intoxication. Second, in vitro evidence indicates that warming is more effective in disordering membranes than in causing intoxication. On the basis of a quantitative analysis of the effects of temperature and ethanol on fluidization, Dr. Goldstein suggests that we need to identify membrane sites that are 10 times more sensitive to ethanol-induced fluidization than they are to temperature-induced increases in fluidization. As yet, such sites have not been identified.

Overall, the relatively powerful ability of small increases in temperature to increase membrane fluidity indicates that the bulk membrane cannot be the critical site at which ethanol acts. Rather, if ethanol acts by increasing fluidization, it has to act on sensitive microenvironment(s) within the membrane.

Conclusion

Dr. Goldstein concludes that the evidence supporting the membrane hypothesis is not convincing at this time. One of the problems in obtaining evidence is our lack of knowledge regarding the key sites of action within the membrane.

Questions

What Is Ethanol's Mechanism of Action Leading To Intoxication?

Dr. Goldstein's presentation clearly points out that membrane fluidity theories are viable but have not been established. A second mechanism, which was not discussed, suggests that ethanol acts directly on proteins, perhaps via interaction with a hydrophobic pocket (Franks and Lieb 1985).

Does Ethanol Act by One or More Mechanism to Cause the Myriad Behavioral Changes Associated With Intoxication?

The membrane fluidity hypothesis suggests that all intoxicating effects of ethanol result from a single mechanism, fluidization of membrane lipids. But membrane fluidity has not been proven as a mechanism, let alone as the only mechanism. Other mechanisms are possible. Very little information linking the behavioral effects of ethanol to a common mechanism is available. In the most complex situation, each behavioral effect of ethanol could reflect a different mechanism.

Does Ethanol Act at One or Many Initial Sites?

This is a key question. As reviewed by Dr. Goldstein, the available evidence strongly indicates that ethanol acts at a hydrophobic site. But it is not clear whether this site is lipid or protein. Furthermore, as indicated by Dr. Goldstein, the finding of differential sensitivities to ethanol-induced fluidization of membrane lipid microenvironments suggests that there may be more than one lipid site of action. The same argument could be made for protein sites. Consequently, it is possible that ethanol could act by one mechanism at a single critical site, by one mechanism at several initial hydrophobic sites, or by more than one mechanism at more than one site.

Mechanism of Action of Ethanol: Evidence From Physical Manipulations

Our laboratory has been using physical manipulations to address some of these questions regarding the mechanism of action of ethanol. Selected results from our and other laboratories are summarized below.

Body Temperature Manipulation

As discussed by Dr. Goldstein, increasing the temperature of membrane preparations *in vitro*, like ethanol, increases membrane fluidity. Therefore, if membrane fluidity changes mediate ethanol-induced intoxication, then sensitivity to the intoxicating effects of ethanol should vary directly with the temperature of the brain during intoxication. We reasoned that the use of a behavioral assay for the temperature-ethanol interaction would be a useful first step to test this prediction while avoiding the technical difficulty of isolating, identifying, and testing critical microenvironments. The general experimental protocol involved using ambient temperature to manipulate the body temperature of mice or rats during intoxication. Typically, intoxicated body temperatures were manipulated between 38 and 32 °C.

EXPERIMENTAL RESULTS

Selected experimental results are summarized in table 2. In general, predictions based on membrane perturbation theories held. Brain sensitivity to acute ethanol-induced intoxication and lethality decreased as body temperature decreased, using several measures of intoxication in subjects representing a variety of genetic backgrounds (Alkana et al. 1985, 1988, 1989; Finn et al. 1986; Grieve and Littleton 1979; Malcolm and Alkana 1981, 1983; Pohorecky and Rizek 1981). Chronic functional tolerance (Alkana et al. 1987*a,b*) and physical dependence (Becker and Randall unpublished data) developed more rapidly at normal than at low body temperatures, suggesting that decreasing the temperature of the membranes decreased the effective dose of ethanol to which tolerance and dependence developed. These temperature-induced changes in ethanol sensitivity did not reflect changes in the pharmacokinetics of ethanol.

However, recent findings suggest that there are exceptions to the general findings summarized above. Specifically, brain sensitivity to ethanol in LS and 129/J mice appeared to decrease as body temperature increased (Alkana et al. 1989). At this time, we do not know the importance of these exceptions.

Table 2.—Ethanol intoxication: Effects of body temperature manipulation in homeotherms

Subject	Measure	Sensitivity change with decreased temperature
Rats		
Long Evans	LORR ^a	↓
Sprague-Dawley	Swimming	↓
Mice		
C57BL/6	LORR	↓
	Rotarod	↓
	Lethality	↓
	Tolerance development	↓
	Dependence development	↓
BALB/c	LORR	↓
DBA/2	LORR	↓
A/He	LORR	↓
SS	LORR	↓
	Lethality	↓
LS	LORR	↑
	Lethality	↓
129	LORR	↑

^aLoss of righting reflex (duration and blood or brain ethanol concentration at the return of the righting reflex).

CONCLUSIONS

Overall, the results of temperature-ethanol interaction studies support the hypothesis that membrane perturbation represent the initial action of ethanol leading to acute intoxication, tolerance, and physical dependence. These results suggest that one, temperature-dependent action is responsible for the acute and chronic behavioral effects of ethanol that were tested. The findings do not provide insight as to whether one or multiple initial sites of action are involved.

The results in LS and 129 mice do not fit the predictions based on perturbation theories. If further studies show that these exceptions are representative of a large population, then the results call into question the membrane fluidization theory. If further tests indicate that the LS and 129 mice are anomalies, then the findings suggest that LS and 129 mice may differ from other animals in temperature-ethanol interactions at a critical sites or sites. Such difference might be useful in identifying these critical sites.

Increased Atmospheric Pressure (Hyperbaric exposure)

Hyperbaric exposure has been shown to reverse general anesthesia and the membrane-perturbing effects of general anesthetics (Johnson and Flagler 1950; Lever et al. 1971). Available evidence suggests that pressure offsets the initial perturbing action of ethanol or squeezes ethanol out of the critical site(s) (Franks and Lieb 1982; Miller 1986). If ethanol acts by perturbing membranes, then hyperbaric exposure should antagonize the intoxicating effects of ethanol.

In the experiments summarized below, experimental animals were administered ethanol before or during exposure to hyperbaric helium-oxygen gas mixtures. Typically, the pressure was 12 atm absolute (ATA). Control animals were exposed to air or helium-oxygen at 1 ATA.

Table 3.—Ethanol intoxication: Effects of hyperbaric exposure

Subject	Measure	Antagonism
Rats		
Sprague-Dawley	Locomotor depression	Yes
Mice		
C57BL/6	LORR ^a	Yes
	Locomotor depression	Yes
	Tolerance development	Yes
	Dependence development	Yes
BALB/c	LORR	Yes
DBA/2	Locomotor activation	Yes
CFW(SW)BR	Aggression depression	Yes
LS	LORR	Yes
SS	LORR	No

^aLoss of righting reflex (duration and blood or brain ethanol concentration at the return of the righting reflex).

EXPERIMENTAL RESULTS

In acute studies (table 3), hyperbaric exposure antagonized the effects of ethanol on a variety of measures in animals representing a host of genetic backgrounds (Alkana and Malcolm 1982; Alkana et al. 1986; Garcia-Cabrera and Berge 1988; Malcolm and Alkana 1982; Syapin et al. 1986, 1988). In addition, hyperbaric exposure during chronic ethanol exposure attenuated the development of chronic functional ethanol tolerance and physical dependence, and it precipitated and exacerbated withdrawal in ethanol-dependent animals (Alkana et al. 1987*a,b*) (table 3). These results could not be explained by changes in ethanol pharmacokinetics or body temperature during intoxication. In contrast to results for other animals tested, recent preliminary results suggest that hyperbaric exposure does not antagonize ethanol-induced loss of the righting reflex in SS mice.

CONCLUSIONS

Overall, the antagonistic effect of pressure against ethanol intoxication is consistent with a membrane fluidization mechanism. The acute studies suggest a common pressure-reversible mechanism for ethanol-induced LORR, stimulation and depression of locomotor activity, and depression of aggressive behavior. The chronic studies suggest that chronic functional ethanol tolerance and physical dependence reflect adaptations to the initial, pressure-reversible actions of ethanol. The results do not provide insight as to whether ethanol acts on single or multiple sites.

The results in SS mice pose a problem, since they do not fit predictions based on perturbation theories or the results in animals representing other genetic backgrounds. If further studies across a broad spectrum of genetic backgrounds using other measures of intoxication show that the lack of hyperbaric antagonism in SS mice is representative of a large population, then the results in SS mice question perturbation theories. On the other hand, if the results in SS mice represent an exception to the rule, then the findings suggest that SS mice may have been selected for resistance to ethanol at a pressure-sensitive site.

Conclusions

Overall, the behavioral studies using physical manipulations agree with in vitro evidence supporting membrane perturbation theories. Collectively, this work suggests that there may be a single, temperature-dependent, pressure-reversible mechanism leading to acute intoxication, tolerance, and dependence. However, the available information does not address the question of whether there is a single critical site or multiple sites of action for ethanol.

In contrast, the results in the LS, SS, and 129 mice suggest that a simple membrane perturbation model may not be appropriate and that there may be different mechanisms of action or critical sites of action for ethanol in animals with different genetic backgrounds.

References

- Alkana, R.L.; Bejanian, M.; Jamieson, B.; Jones, B.; Galleisky, G.G., Syapin, P.J., and Finn, D.A. Temperature dependence of ethanol depression measured by rotarod performance in mice. *Soc Neurosci Abstr* 11:295, 1985.
- Alkana, R.L.; Bejanian, M.; Jones, B.L.; Syapin, P.J.; Crabbe, J.C.; and Finn, D.A. Body temperature manipulation influences genetically determined differences in ethanol sensitivity. In: Kiianmaa, K.; Tabakoff, B.; and Saito, T., eds. *Genetic Aspects of Alcoholism*. Vol. 37. Helsinki: Finnish Foundation for Alcohol Studies, 1989.
- Alkana, R.L.; Bejanian, M.; Syapin, P.J.; and Finn, D.A. Chronic functional ethanol tolerance in mice influenced by body temperature during intoxication. *Life Sci* 41:413-420, 1987a.
- Alkana, R.L.; DeBold, J.R.; Chen, J.; Finn, D.A.; and Syapin, P.J. Ethanol-induced depression of aggressive behavior in mice is antagonized by hyperbaric exposure. *Soc Neurosci Abstr* 12:284, 1986.
- Alkana, R.L.; Finn, D.A.; Bejanian, M.; and Crabbe, J.C. Genetically determined differences in ethanol sensitivity influenced by body temperature during intoxication. *Life Sci* 43:1973-1982, 1988.
- Alkana, R.L., and Malcolm, R.D. Hyperbaric ethanol antagonism in mice: Studies in oxygen, nitrogen, strain and sex. *Psychopharmacology* 77:11-16, 1982.
- Alkana, R.L.; Syapin, P.J.; Galleisky, G.G.; and Finn, D.A. Hyperbaric exposure acts as an ethanol antagonist: Evidence from chronic studies. *Alcohol Alcoholism Suppl* 1:417-421, 1987b.
- Finn, D.A.; Boone, D.C.; and Alkana, R.L. Temperature dependence of ethanol depression in rats. *Psychopharmacology* 90:185-189, 1986.
- Franks, N.P., and Lieb, W.R. Molecular mechanisms of general anesthesia. *Nature* (London) 300:487-493, 1982.

- Franks, N.P., and Lieb, W.R. Mapping of general anaesthetic target sites provides a molecular basis for cutoff effects. *Nature* (London) 316:349-351, 1985.
- Garcia-Cabrera, I., and Berge, O.-G. Pressure reversal of the depressant effect of ethanol on spontaneous behavior in rats. *Pharmacol Biochem Behav* 29:133-141, 1988.
- Goodman, A.G.; Goodman, L.S.; Rall, T.W.; and Murad, F. *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. 7th ed. New York: Macmillan, 1985.
- Grieve, S.J., and Littleton, J.M. Ambient temperature and the development of functional tolerance to ethanol by mice. *J Pharm Pharmacol* 3:707-708, 1979.
- Johnson, F.H., and Flagler, E.A. Hydrostatic pressure reversal of narcotics in tadpoles. *Science* 112:91-92, 1950.
- Lever, M.J.; Miller, K.W.; Paton, W.D.M.; and Smith, E.F. Pressure reversal of anesthesia. *Nature* (London) 231:368-371, 1971.
- Malcolm, R.D., and Alkana, R.L. Temperature dependence of ethanol depression in mice. *J Pharmacol Exp Ther* 217:770-775, 1981.
- Malcolm, R.D., and Alkana, R.L. Hyperbaric ethanol antagonism: Role of temperature, blood and brain ethanol concentrations. *Pharmacol Biochem Behav* 16:341-346, 1982.
- Malcolm, R.D., and Alkana, R.L. Temperature dependence of ethanol lethality in mice. *J Pharm Pharmacol* 35:306-311, 1983.
- Miller, K.W. Are lipids or proteins the target of general anaesthetic action? *Trends Neurosci* 9:49-51, 1986.
- Pohorecky, L.A., and Rizek, A. Biochemical and behavioral effects of acute ethanol in rats at different environmental temperatures. *Psychopharmacology* 72:205-209, 1981.

Syapin, P.J.; Chen, J.; Finn, D.A.; and Alkana, R.L. Antagonism of ethanol-induced activation of mouse locomotor activity by hyperbaric exposure. *Soc Neurosci Abstr* 12:284, 1986.

Syapin, P.J.; Chen, J.; Finn, D.A.; and Alkana, R.L. Antagonism of ethanol-induced depression of mouse locomotor activity by hyperbaric exposure. *Life Sci* 43:2221-2229, 1988.

Discussion

PERIS: It is confusing when one thinks of how the membrane perturbation theory might work in vivo because ethanol also causes hypothermia, which should offset the membrane fluidizing effect of ethanol.

ALKANA: Certainly the effects of temperature are greater than the membrane effects of ethanol. However, they may be localized effects of ethanol that are greater than those that we can measure with our current techniques.

COLLINS: If we look at the data that John Crabbe gave on his HOT and COLD mice, you see that the temperature differential is about the same as for the LS and SS mice. We have determined that there is a linear correlation of the lipid solubility and the differential effect of a series of compounds on sleep time in the SS and LS mice. Yet when we try to correlate the lipid solubility with temperature effect of these compounds, we find that there is no correlation.

Ethanol and the Sodium Channel

*Walter A. Hunt*¹

Introduction

Since the demonstration in 1846 that ether could be used successfully to induce surgical anesthesia, the question of how anesthetics exert their action has been under study. The belief today is that anesthetics are effective because of their interaction with biologic membranes. The discovery at the turn of the century by Meyer (1899) and Overton (1896), using aliphatic alcohols, that the potency of anesthetics is directly related to their ability to partition into membranes has provided the basis for a great deal of research, including studies into the mechanisms of action of ethanol.

Although a number of studies have addressed the role of alcohol-membrane interactions as a site of action of ethanol, the important breakthrough occurred with the publication of results of experiments using molecular probes, whose properties are studied by using the techniques of electron spin resonance spectrometry and fluorescence spectrophotometry. These results demonstrated that ethanol significantly disorders the lipids of synaptosomal membranes in a dose-dependent manner and in concentrations encountered after consumption of alcoholic beverages (Chin and Goldstein 1977; Harris and Schroeder 1981). In addition, the potency of aliphatic alcohols to disorder membranes is directly related not only to their lipid-water partition coefficient but also to their potency as central nervous system (CNS) depressants.

¹Behavioral Sciences Department, Armed Forces Radiobiology Research Institute, Bethesda, MD 20814-5145.

Since membranes provide largely a support function in cells, the mode by which disordering of membranes could alter behavior depends on the disruption of the molecular entities that are responsible for electrical conduction along neurons and synaptic transmission between neurons. Although ethanol could (and probably does) act on a variety of these entities, a potential target fundamental to the functioning of the brain is the process by which electrical impulses are generated and propagated. From the pioneering work of Hodgkin and Huxley (1952), it is known that electrical impulses are generated through transient changes in the movement or conductance of ions (especially sodium) carrying an electrical charge through the neuronal plasma membrane. More recent research in a number of laboratories has indicated that ions move through channels in the membrane specific for a given ion. One important channel is the sodium channel.

The sodium channel conducts sodium through the membrane in response to changes in the voltage gradient across the membrane. As the gradient is reduced during depolarization, the membrane, which normally does not conduct sodium ions, becomes transiently permeable to them. How the sodium channel opens and closes is not yet understood, but studies using neurotoxins have provided valuable information on the molecular mechanisms involved. Both electrophysiologic and biochemical approaches have proved useful. Such approaches have also shed light on a possible action of ethanol on the sodium channel.

Effects of Ethanol In Vitro on Sodium Channels

The earliest studies to examine the effect of ethanol on the sodium channel used electrophysiologic techniques on invertebrate neurons. In these studies, high concentrations of ethanol reduced the height of the action potential and the maximum sodium conductance (Armstrong and Binstock 1964; Moore et al. 1964). Similar results were obtained with electrically stimulated brain slices. In these experiments, 105 mM ethanol inhibited the uptake of sodium into intracellular spaces (Wallgren et al. 1974).

More recent research on the effects of ethanol on sodium channels has taken advantage of the latest findings involving the molecular characterization of the channel. The sodium channel appears to be a glycoprotein with multiple

polypeptide subunits that traverse the neuronal membrane (Catterall 1980). In addition, sodium channels have an absolute requirement for lipids to exhibit optimal activity (Tamkun et al. 1984).

Binding sites on the channel selective for neurotoxins have been shown electrophysiologically to modify the activity of the sodium channel. There appear to be at least three sites (Catterall 1980). Site I is located on the outer surface of the neuronal membrane. This is the site on which toxins such as tetrodotoxin and saxitoxin block the generation of an action potential. Site II, located in the lipid (hydrophobic) core of the membrane, binds the toxins veratridine and batrachotoxin. These toxins selectively open the sodium channel. And site III, located on the membrane surface with projections to the lipid core, apparently serves a modulatory role. Toxins such as scorpion venom and sea anemone toxin block inactivation of the channel and enhance the activity of toxins that interact with site II. These sites have been exploited to gain further knowledge about the interaction of ethanol with sodium channels.

The main studies measured the rate of entry of ^{22}Na into synaptosomes that were exposed to veratridine or batrachotoxin (Mullin and Hunt 1984; 1985; Harris and Bruno 1985). Initial findings indicated that incubation of synaptosomes with ethanol (25-500 mM) resulted in a concentration-dependent reduction in the initial rate of sodium uptake with incubation times of less than 10 s. No effect on sodium uptake in the absence of neurotoxins was observed. Inhibition of sodium uptake occurred at sublethal, pharmacologically relevant concentrations and was fully reversible when the ethanol was removed from the membranes by centrifugation and washing. When concentration-response curves were constructed for veratridine and batrachotoxin, the maximum effect of the toxins was reduced, suggesting an action on site II of the sodium channel.

Subsequent experiments were designed to determine on which of the three sites on sodium channels ethanol exerted its action (Mullin and Hunt 1985, 1987). Site I was examined by measuring the relative effectiveness of tetrodotoxin to block sodium uptake and the binding of ^3H -saxitoxin. Tetrodotoxin inhibited sodium influx in a concentration-dependent manner. Addition of ethanol (200 mM), to the medium had no effect on the action of tetrodotoxin. When the ability of ^3H -saxitoxin to bind to site I was assessed

in the presence and absence of ethanol (400 mM), again no effect was observed.

Because of its location in the lipid core of the membrane, site II was initially studied by assessing the potency of various aliphatic alcohols and anesthetics with different lipid solubilities. When these drugs were tested, the potency to inhibit neurotoxin-induced sodium uptake was directly related to the lipid-water partition coefficient and the potency to disorder membranes (Mullin and Hunt 1985; Harris and Bruno 1985). The other approach to studying site II was to analyze the binding of ^3H -batrachotoxin, as described in a later section.

To study site III, synaptosomes were incubated with scorpion venom. Scorpion venom does not have an action of its own on sodium influx but will enhance the action of batrachotoxin through an allosteric mechanism (Tamkun and Catterall 1981). The addition of ethanol (200 mM) inhibited batrachotoxin-induced sodium influx to the same extent in both the presence and absence of scorpion venom (Mullin and Hunt 1985).

The results presented above suggest that sodium channels are sensitive to inhibition by ethanol in concentrations corresponding to those that induce behavioral intoxication. The site of action of ethanol appears to be in the hydrophobic core of the neuronal membrane.

Effects of Ethanol In Vivo on Sodium Channels

Since it is important to understand how the sodium channels respond to the presence in ethanol in vivo, animals were given various doses of ethanol orally, and the response of the channel to batrachotoxin and added ethanol in vitro was determined at different times thereafter (Mullin et al. 1987). Ethanol doses of 3-6 g/kg were ineffective in altering batrachotoxin-induced sodium uptake into synaptosomes. This lack of effect may have been due to the absence of ethanol in the tissue preparation at the time uptake was measured. However, when 400 mM ethanol was added in vitro, the inhibition of sodium uptake was progressively less into synaptosomes from animals receiving increasingly higher doses of ethanol, suggesting the development of an acute tolerance. Blood ethanol concentrations ranged from 47 to 75 mM.

This apparent tolerance to the inhibitory effect of ethanol *in vitro* on the sodium channel lasted as long as ethanol was present in the blood.

Since chronic exposure to ethanol can result in tolerance and physical dependence, experiments were undertaken to determine whether such conditions might influence the activity of the sodium channel. Physical dependence was induced by the method of Majchrowicz (1975), whereby rats are given oral ethanol doses of 9-11 g/kg/day in multiple fractions for 5 days. In contrast to responses found after a single dose of ethanol, after 2 and 5 days of ethanol treatment, batrachotoxin-stimulated sodium uptake was inhibited (Mullin et al. 1987). This effect lasted at least 5 days after withdrawal but disappeared by 10 days. When 400 mM ethanol was added *in vitro*, the normal inhibitory effect was diminished, like that found after a single dose of ethanol. However, this effect was maintained long after the elimination of ethanol from the blood, lasting at least 20 days after withdrawal but recovering by 35 days. Tolerance to the lipid-disordering effect of ethanol also followed this time course.

The results of the *in vivo* studies indicate that tolerance develops rapidly to the inhibitory effect of ethanol *in vitro* on the batrachotoxin-stimulated sodium uptake. The rate of recovery of this tolerance depends on the duration of ethanol treatment. After a single dose of ethanol, the tolerance dissipates as the ethanol is eliminated from the blood. However, as more ethanol is administered, the time to recover increases substantially. After 5 days of ethanol administration, recovery takes 3-4 weeks.

Effect of Ethanol on Batrachotoxin Binding

Disruption of neurotoxin-stimulated sodium uptake by ethanol could result from disturbances in two fundamental processes: (1) the ability of the neurotoxin to properly interact with its receptor and (2) the molecular processes that occur to increase sodium influx. To test the first possibility, the ability of ^3H -batrachotoxin A 20- α -benzoate (BTX-B) to bind to synaptosomes was studied. It was presumed that if the binding of ^3H -BTX-B is unaffected by ethanol exposure, the likely site of action of ethanol involves the second possibility.

Ethanol *in vitro* progressively decreased BTX-B binding when the concentration of ethanol exceeded 75 mM (Mullin and Hunt 1987). This effect resulted from a reduced affinity of the BTX-B for its receptor site rather than from a lower number of active binding sites. The reduced affinity was due to a slower rate of dissociation of the BTX-B from its receptor. Acute or chronic administration of ethanol had no effect on binding or on the effect of ethanol *in vitro*.

Although ethanol exposure will reduce the binding of batrachotoxin to its receptor, this action alone does not appear to be responsible for the ethanol-induced inhibition of neurotoxin-stimulated sodium influx. The characteristics of each effect are quite different. Inhibition by ethanol of sodium uptake reflects a loss of the maximum effectiveness of the neurotoxin, whereas inhibition of batrachotoxin binding results from a reduced affinity for the receptor. Also, the affinity was determined in the presence of scorpion venom, which increases affinity. A single dose of ethanol induced a reversible state of tolerance to the inhibitory effect of ethanol *in vitro* on sodium uptake. No such effect on batrachotoxin binding was observed. Finally, chronic administration of ethanol decreased sodium uptake in the absence of ethanol *in vitro* and induced a state of tolerance to added ethanol for over 3 weeks. Again, no effect on batrachotoxin binding was observed.

Conclusions

Criteria for a putative mechanism of action for ethanol on the brain include the following (Hunt 1985): "(1) Biochemical and biophysical changes induced by alcohols have to occur at the sublethal concentrations found *in vivo* after ethanol ingestion [100 mM (460 mg/dl)] and in physiologically important magnitudes to be considered relevant. (2) The time course of these changes must correlate with the appearance and disappearance of CNS depression and with changes in blood (brain) alcohol levels, since the time course of depression corresponds with these changes. (3) The increasing potency of alcohols with increasing lipid solubility must be explained. And (4) the changes involved must be relevant to mechanisms responsible for neuroexcitability."

The effects of ethanol on sodium channels conform to a large extent to the criteria just stated. The movement of sodium ions across the synaptosomal membrane *in vitro* is reduced in concentrations of ethanol found after consumption of alcoholic beverages. This action is reversible and can be expected to comply with criterion 2. Various other aliphatic alcohols inhibit sodium uptake with potencies that correlate with their lipid solubilities. An interaction with the lipid core of the membrane (site II) provides the best explanation for how ethanol acts on the channel. Finally, the sodium channel is an important component of the process of generating and propagating electrical potentials along neurons.

The role of sodium channels in the chronic effects of ethanol is not clear. The channel will respond to the chronic presence of ethanol by becoming tolerant to its disruption of channel function. The time course of this tolerance depends on how long the ethanol is given. After a single dose, the tolerance to the effect of ethanol *in vitro* follows the presence of ethanol in the blood. After 5 days of treatment, however, the tolerance lasts over 3 weeks. It is probable that these actions are a consequence rather than a cause of tolerance.

Ethanol has a multitude of actions on the body, largely because of the nonspecific nature of its interaction with various components of the cell. Thus, ethanol might not be expected to have a single mechanism of action. Even if a primary action of ethanol is a disruption of the lipid environment of membranes, the consequences of this action may be numerous, including dysfunction of the sodium channel. An action on the sodium channel is probably not the only one that contributes to the behavioral effects of ethanol. It is likely that a combination of different mechanisms, when taken together, induce the phenomenon we call ethanol intoxication.

References

- Armstrong, C.M., and Binstock, L. The effects of several alcohols on the properties of the squid giant axon. *J Gen Physiol* 48:265-277, 1964.
- Catterall, W.A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu Rev Pharmacol Toxicol* 20:15-43, 1980.

- Chin, J.H., and Goldstein, D.B. Effects of low concentrations of ethanol on the fluidity of spin-labeled erythrocyte and brain membranes. *Mol Pharmacol* 13:435-441, 1977.
- Harris, R.A., and Bruno, P. Effects of ethanol and other intoxicant-anesthetics on voltage-dependent sodium channels of brain synaptosomes. *J Pharmacol Exp Ther* 232:401-406, 1985.
- Harris, R.A., and Schroeder, F. Ethanol and the physical properties of brain membranes: Fluorescence studies. *Mol Pharmacol* 20:128-137, 1981.
- Hodgkin, A.L., and Huxley, A.F. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol (London)* 117:500-544, 1952.
- Hunt, W.A. *Alcohol and Biological Membranes*. New York: Guilford Press, 1985.
- Majchrowicz, E. Induction of physical dependence upon ethanol and its associated behavioral changes in rats. *Psychopharmacologia* 43:245-254, 1975.
- Meyer, H. Welche Eigenschaft der Anästhetica bedingt ihre narkotische Wirkung? *Naunyn-Schmiedeberg's Arch Exp Pathol Pharmacol* 42:109-118, 1899.
- Moore, J.W.; Ulbricht, W.; and Takata, M. Effect of ethanol on the sodium and potassium conductances of the squid axon membrane. *J Gen Physiol* 48:279-295, 1964.
- Mullin, M.J.; Dalton, T.K.; Hunt, W.A.; Harris, R.A.; and Majchrowicz, E. Actions of ethanol on voltage-sensitive sodium channels: Effects of acute and chronic ethanol treatment. *J Pharmacol Exp Ther* 242:541-547, 1987.
- Mullin, M.J., and Hunt, W.A. Ethanol inhibits veratridine-stimulated sodium uptake in synaptosomes. *Life Sci* 34:287-292, 1984.

- Mullin, M.J., and Hunt, W.A. The actions of ethanol on voltage-sensitive sodium channels: Effects on neurotoxin-stimulated sodium uptake in synaptosomes. *J Pharmacol Exp Ther* 232:413-419, 1985.
- Mullen, M.J., and Hunt, W.A. Actions of ethanol on voltage-sensitive sodium channels: Effects on neurotoxin binding. *J Pharmacol Exp Ther* 242:536-540, 1987.
- Overton, E. Über die osmotischen Eigenschaften der Zelle in ihrer Bedeutung für die Toxikologie und Pharmakologie. *Z physikalische Chemie* 22:189-209, 1896.
- Tamkun, M.M., and Catterall, W.A. Ion flux studies of voltage-sensitive sodium channels in synaptic nerve-ending particles. *Mol Pharmacol* 19:78-86, 1981.
- Tamkun, M.M.; Talvenheimo, J.A.; and Catterall, W.A. The sodium channel from rat brain: Reconstitution of neurotoxin-activated ion flux and scorpion toxin binding from purified components. *J Biol Chem* 259:1676-1688, 1984.
- Wallgren, H.; Nikander, P.; von Boguslawsky, P.; and Linkola, J. Effects of ethanol, tert. butanol and clomethiazole on net movements of sodium and potassium in electrically stimulated cerebral tissue. *Acta Physiologica Scandinavica* 91:83-93, 1974.

Discussion

LESLIE: A number of laboratories have established that ethanol inhibits calcium channels. An interesting feature of ethanol's effects on calcium channels is that the potency of its inhibitory effects seems to depend upon the brain region studied and the calcium channel type. Our studies have shown that calcium entry into synaptosomes from cerebral cortex is inhibited by ethanol concentrations as low as 25 mM. Calcium entry into striatal synaptosomes, on the other hand, measured under identical experimental conditions, was inhibited only by high concentrations of ethanol above 100 mM. Tolerance development to calcium channel inhibition after chronic

ethanol treatment also depends upon the brain region studied. Calcium channels from cerebrocortical synaptosomes adapt to chronic ethanol, while synaptosomes from striatum do not. Along the same lines, recent work from Llinas' laboratory has shown that octanol inhibits apparently identical, low-threshold calcium channels to different degrees, depending upon the brain region studied. For example, they report that 1 μM octanol almost completely inhibited low-threshold calcium channels in inferior olive. On the other hand, 25 μM octanol only partially inhibited low-threshold calcium channels in the thalamus. Thus, calcium channels which appear to be identical seem to vary in sensitivity to alcohols. It may be that the microenvironment sites around calcium channels vary in different brain regions. Some microenvironment sites may be subject to perturbation by ethanol, while others may not. It is also known that there are distinctly different types of calcium channels in the brain. Thus far, three types have been identified. These are T-, L- and N-type channels. Little is known about the effects of ethanol on the T- and N-type channels, although the low-threshold calcium channel studied in Llinas' laboratory appears to be the T-type channel. L-type channels are inhibited by dihydropyridine calcium channel blockers. Dihydropyridine calcium channel blockers potentiate the sedative properties of ethanol. Furthermore, chronic ethanol treatment results in an increase in dihydropyridine binding sites in the brain. Thus, ethanol may interact with L-type calcium channels to produce its effects. A word of caution, however. The concentrations of the dihydropyridine calcium channel blockers used in these studies have been quite large. One could question the calcium channel specificity of the doses used.

SIGGINS: I am a little concerned because the electrophysiology that has attempted to record ionic currents directly has generally shown that over 100 mM ethanol is required to block Na^+ channels, that is, those Na^+ channels that have to do with the action potential. Also, high concentrations of ethanol are required to block Ca^{2+} channels that are associated with the spike. Of course, as you point out, multiple channel types may help explain some of the divergence between electrophysiology and biochemistry. Some investigators (Oakes and Pozos) find effects of ethanol on Ca^{2+} channels at 100 mM or less in dorsal root ganglia, but others (Weight and co-workers), using voltage clamp techniques in the hippocampus, have difficulty finding effects at 100 mM or more. The Na^+ channel is another problem as there is also more than one type. One is tetrodotoxin (TTX) sensitive and another is

slower and is not TTX sensitive. I don't know how to compare these. The upward stroke of the action potential is related to the inward flow of Na^+ , but whether that bears any relationship to the TTX influx is not clear. What kind of channel are you dealing with, Walter?

HUNT: It is an important point that George is raising. There is a link of a "so what" question. If you can see these changes, is that enough to mean anything? It would be nice to see that the biochemical changes that we can measure are correlated with some electrophysiologic event. It is difficult to get any data except in the cerebral cortex because the activity of the sodium channel in other areas of the brain is low. Different brain areas vary in this sensitivity of ethanol.

LESLIE: What do you think about the Llinas' finding that low-threshold calcium channels differ in sensitivity to octanol, depending upon the brain region studied?

SIGGINS: That is the same as the so-called transient of T channel and the function of that is not entirely known. Whether it is involved either in ethanol intoxication or in carrying much current in the spike is, at this point, unclear.

It takes a lot more ethanol to intoxicate than it does to inhibit the transient Ca^{2+} current; that is in the 1 mM range. No one has gone beyond what Llinas has shown, trying to relate what the ethanol does, to what the transient Ca^{2+} channel really does functionally. I believe he thinks that it has something to do with coupling cells together electrically and in fact may have more to do with inhibition (via the Ca^{2+} -dependent K^+ currents) and less to do with spiking.

VOGEL: What does it really mean? I know that you cannot answer that, but can we speculate what blockade of the sodium channel or changes in the sodium channel would do. Would this mean a difference in conductance; would it mean decreased uptake in neurotransmitters or response to neurotransmitters?

HUNT: Only to a limited extent, since we are comparing different systems and these studies are always studied in isolation. But a simple speculation

would be that since transmitter release is a result of a voltage-dependent process, if you had blockade of sodium and calcium channels, we would expect a reduction in transmitter release. But that in itself may not have much bearing on behavior, since all of these effects act in concert.

HARRIS: A recent article in *Nature* reports a study of potassium channel in snails, where neurons could be identified and were identical until halothane was added. One channel was completely inhibited by halothane, but the one next to it was unaffected. Therefore, there is a heterogeneity in neurons that will be very difficult to study in anything more complex than the snail.

Electrophysiological Evaluation of Acute Ethanol Effects on Transmitter Responses in Central Neurons¹

*George R. Siggins, S. G. Madamba, and
S. D. Moore²*

Introduction

The mechanisms of the acute action of ethanol on central nervous function are poorly understood, despite evidence that ethanol profoundly alters behavior and neuronal excitability (Berry and Pentreath 1980; Bloom and Siggins 1987; Faber and Klee 1977; Grenell 1982; Kalant 1974; Siggins and Bloom 1981; Siggins et al. 1987*a*; Suzdak et al. 1986). At the cellular level, both excitatory and inhibitory effects are seen (Berger et al. 1982; Berry and Pentreath 1980; Kalant 1974; Siggins and Bloom 1981; Siggins et al. 1987*a*). A major question is whether ionic channels are specifically altered by ethanol (Oakes and Pozos 1982; Hunt this volume). The synapse seems to be a neuronal site most sensitive to ethanol (Faber and Klee 1977; Kalant 1974; Siggins et al. 1987*a,b*; Strong and Wood 1984). Therefore, our studies have

¹The original work cited here was supported by grants AA 06420 and AA 07456 from the National Institute on Alcohol Abuse and Alcoholism and grant MH 44346 from the National Institute of Mental Health.

²Alcohol Research Center, Research Institute of Scripps Clinic, La Jolla, CA 92037.

emphasized interactions of ethanol with synaptic activity and transmitter effects examined at the cellular level, using several *in vivo* and *in vitro* preparations. Of particular interest was the possibility that electrophysiologic responses to the inhibitory transmitter gamma-aminobutyric acid (GABA) might be enhanced by ethanol or that ethanol might itself exert a GABAergic action, as suggested by the results of biochemical experiments (Suzdak et al. 1986; Harris and Allan this volume; Ticku et al. this volume).

In Vivo Electrophysiologic Models: Extracellular Recording

Cerebellum

We (Bloom et al. 1984; Siggins and Gruol 1986) have studied the effect of systemic ethanol on extracellular responses of identified Purkinje cells to GABA and norepinephrine, two strong candidates for inhibitory transmitters to these cells (Bloom et al. 1984). To date, we have not seen evidence of changes in the responsiveness of these cells to either transmitter at systemic doses of 0.5-2 g/kg (Bloom et al. 1984). Furthermore, the inhibitory pause recorded from Purkinje cells following local surface stimulation of the cerebellum is, if anything, reduced by systemic administration of ethanol (Bloom et al. 1984). It is likely that this pause is mediated by GABAergic input to these cells from local inhibitory interneurons (Siggins and Gruol 1986). Furthermore, the predominant response of Purkinje cells to systemic ethanol is a slight increase in spontaneous simple spike firing rate and a dramatic increase in the firing frequency of climbing fiber bursts (Bloom et al. 1984; Rogers et al. 1980). These excitatory effects of sedative doses of ethanol are not consistent with a GABA-like ethanol effect or enhancement of GABA-activated Cl^- flux as a primary mechanism of ethanol-induced sedation (Suzdak et al. 1986; Harris and Allan this volume; Ticku et al. this volume).

Hippocampus

We (Mancillas et al. 1986a) have expanded on these studies of systemic ethanol effects on neuronal responses to transmitters, using extracellular

recording of, and iontophoretic application of transmitters to, hippocampal CA1 and CA3 pyramidal neurons. In brief, we saw no alteration of inhibitory responses to norepinephrine or serotonin at doses of 0.75 to 1.5 g/kg of ethanol intraperitoneally (IP) (Mancillas et al. 1986a). Inhibitory responses to GABA were sometimes apparently enhanced after ethanol injection but no more than was seen after injection of saline alone. Trials with intermittent GABA application from two different barrels of the same multibarrel pipette assembly indicated that GABA responses tended to improve over time without any ethanol administration, probably as a result of long-term "warm-up" of the iontophoresis barrels (Mancillas et al. 1986a). Such iontophoresis artifacts could have been the source of the apparent potentiation of GABA responses in cortical neurons reported after extremely low (milligram per kilogram) doses of systemic ethanol (Nesteros 1980).

In contrast to our negative findings with GABA, responses to two other agonists were enhanced by these systemic doses of ethanol. The inhibitory responses to the peptide somatostatin (SS; somatotropin release inhibitory factor) were significantly increased in a large percentage of pyramidal neurons at 15-40 min after IP administration of 0.75 or 1.5 g/kg of ethanol (Mancillas et al. 1986a). Excitatory responses to acetylcholine (ACh) were similarly enhanced at about the same time after the same doses of ethanol (Mancillas et al. 1986a). By contrast, excitatory responses to iontophoresis of glutamate were unaffected or slightly decreased (see figure 1 of Mancillas 1986a). It may be relevant to the latter observation that recent studies of Lovinger et al. (1989) indicate that low concentrations of ethanol have no effect on GABA current but antagonize the ionic currents elicited by activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor in neurons of hippocampal cultures.

An In Vitro Model: Hippocampal Slice Preparation

Membrane Effects

We have used intracellular current and voltage clamp recording of hippocampal CA1 and CA3 pyramidal neurons of the superfused dorsal hippocampal slice to test the action of defined concentrations of ethanol on membrane properties and transmitter responses (Siggins et al. 1987b). In

current clamp mode, there were only weak and variable effects of ethanol (11-300 mM) on membrane potential, spike size or shape, input resistance, and spike afterhyperpolarizations (AHPs). These data do not support a major action of intoxicating doses of ethanol on the Na^+ and Ca^{2+} ionic channels responsible for resting and action potentials. Our findings also contrast somewhat with the report of Carlen et al. (1982) that ethanol hyperpolarized these hippocampal slice neurons and enhanced the AHPs. However, we often did see reductions in the rate of spontaneous spike firing with low ethanol concentrations, usually associated with no change in membrane potential or with a paradoxical depolarization (Siggins et al. 1987b). These reductions in firing could result from remote effects on upstream neurons projecting to the cell under study.

Effects on Transmitter Mechanisms

Of course, these changes in firing rate also could result from alteration of responsiveness to neurotransmitters. Therefore, we tested the effect of ethanol superfusion on responses to GABA applied to the pyramidal neurons from micropipettes positioned near the recorded cell. There was no consistent effect of 40-50 mM ethanol (equivalent to blood levels of about 200 mg percent) on the magnitude or duration of the hyperpolarizations elicited by local GABA application (Siggins et al. 1987b). Furthermore, 11-100 mM ethanol most often *reduced* the amplitude and duration of inhibitory postsynaptic potentials (IPSPs) evoked by stimulation of the stratum radiatum (Siggins et al. 1987b), in contrast to the report by Carlen et al. (1982) that ethanol enhanced IPSPs in these slice neurons. Since the stratum radiatum-evoked IPSP is mediated by GABA release (Siggins and Gruol 1986), our finding of IPSP reduction in these hippocampal neurons further draws into question the GABA hypothesis of ethanol sedation (Suzdak et al. 1986; Harris and Allan this volume; Ticku et al. this volume), at least for this brain region.

We have also used the hippocampal slice to begin to examine the mechanisms responsible for the ethanol enhancement of SS and ACh responses seen with extracellular recording *in vivo*. These agonists exert opposite actions on central neurons via multiple mechanisms (Bernardo and Prince 1981; Dodd et al. 1981; Gahwiler and Brown 1987; Halliwell and Adams 1982; Inoue et al. 1987; Jacquin et al. 1988; Madison et al. 1987;

Moore et al. 1988; Pittman and Siggins 1981; Scharfman and Schwartzkroin 1988; Siggins and Gruol 1986). Interestingly, our *in vivo* studies showed that SS enhances ACh excitatory effects, although SS by itself is inhibitory (Mancillas et al. 1986b) and hyperpolarizing (Pittman and Siggins 1981). However, research in our laboratory (Jacquin et al. 1988; Moore et al. 1988) and elsewhere (Halliwell and Adams 1982) has shown a common, reciprocal point of interaction: the time- and voltage-dependent K^+ current sustained at slightly depolarized membrane potentials, termed the M-current (I_M). Muscarinic cholinergic agonists decrease this current (Adams et al. 1982; Brown and Adams 1980; Halliwell and Adams 1982), whereas SS analogs (SS14 and SS28) augment it (Jacquin et al. 1988; Moore et al. 1988).

Therefore, as a first approach to determine the mechanism of ethanol's interaction with ACh and SS, we (S. D. Moore, S. G. Madamba, and G. R. Siggins submitted) examined the effects of ethanol on the M-current in hippocampal slices completely immersed in a temperature-controlled recording chamber and superfused with standard artificial cerebrospinal fluid to which drugs and transmitter candidates were added (see Moore et al. 1988 and Siggins et al. 1987b for methods). Tetrodotoxin ($1 \mu M$) was always present to prevent Na^+ spikes and transmitter release. Voltage clamp studies were performed with an Axoclamp preamplifier. In hippocampal pyramidal neurons, the M-current is seen best in voltage clamp mode with holding potentials of minus 40 to minus 50 mV and small hyperpolarizing command steps of 5-25 mV and 700-1,000 ms (Halliwell and Adams 1982), when it appears as a slow inward current relaxation following the instantaneous (ohmic) inward current drop (figures 1 and 2; Adams et al. 1982; Brown and Adams 1980; Halliwell and Adams 1982).

In 36 of 42 neurons recorded with this paradigm, superfusion of 22-44 mM ethanol measurably reduced the amplitude of the M-current relaxations (figure 2). The mean reduction of M-current amplitude by 44 mM in those cells submitted to a standardized -40-mV holding potential ranged from 30 to 42 percent over all command step sizes (average for all steps was 36 percent). Ethanol reduces the M-current equally over the whole range of command sizes and thus does not appear to shift the voltage sensitivity of this current. M-current reduction was at least partially reversible by washout of ethanol and appeared to be maximal at 44 mM ethanol (equivalent to a 200

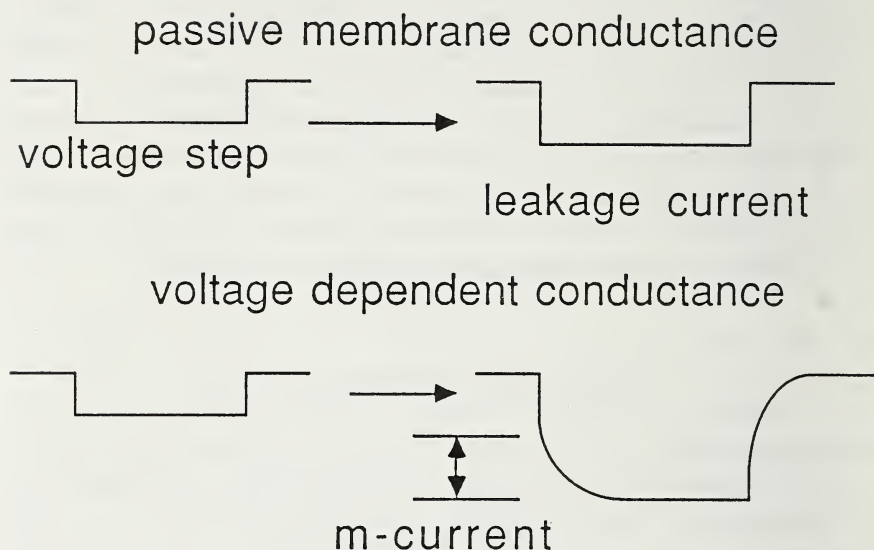


Figure 1.—Schematic of the voltage clamp method of estimating the size of the M-current and the effect of M-channels on membrane current responses. (*Top*) Ohmic current response of a neuronal membrane if only non-voltage-dependent, leakage channels are open (e.g., at normal resting membrane potentials in some neurons). In a cell held at resting potential (upper left voltage trace), a hyperpolarizing step "command" results in the passage of current (upper right trace) through channels that do not close at the new hyperpolarized potential. (*Bottom*) Effect of insertion of M-channels in the membrane when the membrane potential is held at somewhat depolarized levels (e.g., -40 mV). An abrupt hyperpolarizing step now produces an instantaneous ohmic drop in current (due to ions passing through leakage and other channels that have not had time to close), followed by a slowly settling current "relaxation" resulting from the time-dependent closing of M-channels at the new hyperpolarized potential. The difference between the instantaneous current and steady-state current at the end of the hyperpolarizing command constitutes a measure of the M-current amplitude at a given holding potential. Inward currents are down by convention.

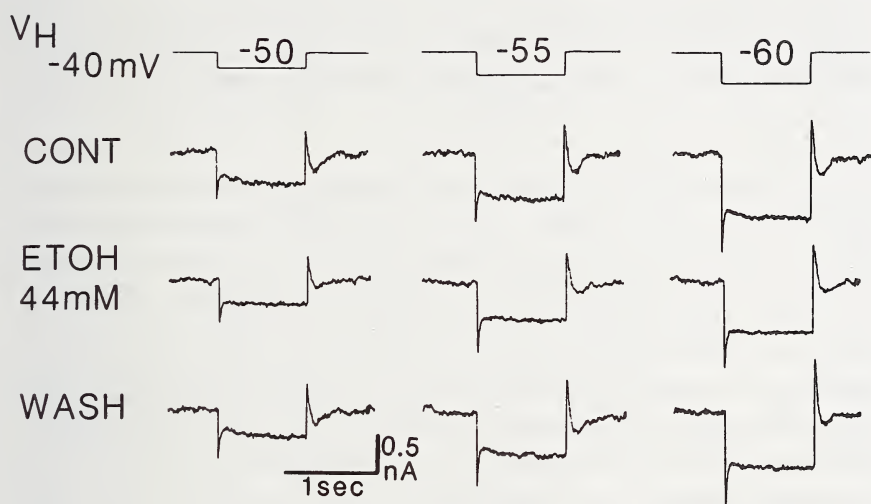


Figure 2.—Effect of 44 mM ethanol on M-current magnitude in hippocampal pyramidal neurons. Shown are representative current responses (bottom three panels) to hyperpolarizing voltage steps to -50, -55, and -60 mV (top panel) from a holding potential (V_H) of -40 mV, obtained before and after 5-8 min of ethanol (EtOH) superfusion and 25 min after washout (wash of ethanol). Note the flattening of the M-current relaxation (accompanied by an inward shift in the holding current that is not shown) and the slight reduction in the ohmic drop (decreased conductance) during ethanol superfusion.

mg percent blood ethanol level); 22 mM ethanol was less effective (nine cells), whereas 11 mM was ineffective in one cell. The effects of 44 mM ethanol on I_M at -40-mV holding potential were most often (11 of 25 cells completely analyzed) accompanied by a net inward holding current (mean = 305 pA) and less often by a weaker outward current or no current change. When held at resting potential, only weak outward currents or no change occurred.

In many respects, ethanol appears to act on these neurons like muscarinic cholinergic agonists (Bernardo and Prince 1981; Halliwell and Adams 1982; Madison et al. 1987; Siggins et al. 1987*a,b*). Since both substances reduce the M-current, ethanol could act by releasing or augmenting the effects of endogenous ACh. However, in five pyramidal cells studied to date, the effects of 44 mM ethanol on the M-current were not altered measurably by superfusion of 1 μ M atropine. In tests of the specificity of this ethanol M-current effect, the inward relaxations attributable to the Q-current (Halliwell and Adams 1982) were not altered by 22 or 44 mM ethanol, nor was the I_{AHP} as previously defined (e.g., Madison et al. 1987) reproducibly altered. In preliminary voltage clamp studies, there did not appear to be a measurable effect of ethanol (44 mM) on I_A , the transient K^+ current. Thus, the ethanol- I_M effect appears to be relatively selective at these ethanol concentrations.

The interaction of ethanol with SS effects seen in vivo (Mancillas et al. 1986*a*) prompted us to seek the mechanisms of this interaction in vitro. Higher concentrations of ethanol (44 mM) that nearly completely block the M-current relaxations continued to do so during concomitant superfusion of concentrations of SS14 (1 μ M) that alone nearly double the magnitude of the relaxations (Moore et al. 1988). However, the M-current-reducing effects of 22 mM ethanol were overcome and reversed to an augmentation by 1 μ M SS14 (data not shown). Thus, at the level of the M-current, the interaction of ethanol and SS appears to be dose dependent.

Discussion

There are several important implications of the ethanol M-current effect. (1) Our negative data with atropine suggest that the ethanol affect does not arise from the release of, or augmentation of the effect of, endogenous ACh, but is

likely exerted "downstream" from the ACh-muscarinic receptor activation, perhaps at the level of second messengers or the ion channel itself (Dutar and Nicoll 1988). (2) The M-current effect of ethanol could explain the excitatory responses of some neurons to ethanol (e.g., Berger et al. 1982; Rogers et al. 1986) and the frequent depolarizations with ethanol superfusion seen in our laboratory with current clamp recordings of CA1 pyramidal neurons (Siggins et al. 1987a). These responding cells may have had resting membrane potentials within the range of M-current activation (usually -60 mV or less), whereas those cells not depolarized may have had more negative resting potentials outside the range. Similarly, contrasting ethanol data taken from the same cell type by different laboratories (Carlen et al. 1982; Siggins et al. 1987a) may derive from differences in the usual resting potentials generated by the different preparation methods and from ethanol actions on such voltage-dependent conductances as the M-current. Thus, ethanol's final effect either in vivo or in vitro may be conditional, depending on the prior excitability and the presence or absence of M-channels in each cell. (3) Ethanol might be expected to increase bursting activity, as the M-current itself tends to prevent prolonged depolarization, clamping the membrane potential at rest (Adams et al. 1982; Brown and Adams 1980). (4) Our data suggest that the interaction of ethanol and SS is more complicated than originally implied by the previous in vivo findings showing ethanol potentiation of SS-evoked discharge inhibition (Mancillas et al. 1986a). The high ethanol concentration (44 mM) generally blocked the SS effect. By contrast, the low concentration (22 mM) equivalent to a 100 mg percent blood ethanol level and thus within the range of systemic levels reached in the in vivo study (Mancillas et al. 1986a), did not reduce the SS effect but also did not seem to potentiate it. Further studies using variable concentrations of SS will be required to further test I_M involvement in the ethanol potentiation of SS effects. Alternatively, other reported membrane sites of SS, ACh, and/or ethanol action, such as CA^{2+} currents (Gahwiler and Brown 1987; Luini et al. 1986; Tsunoo et al. 1986) or an inwardly rectifying K^+ current (Inoue et al. 1987; Mihara et al. 1987; North et al. 1987; Yamashita et al. 1987), may be the primary locus of SS-ethanol interactions and should be examined in the future.

With respect to the effect of SS on an inward rectifying K^+ channel, in many hippocampal pyramidal neurons SS elicits an outward current at membrane holding potentials (-60 to -75 mV) that should be decidedly outside the M-

current activation range (S. D. Moore, S. G. Madamba, and G. R. Siggins, in preparation). Since this SS current is blocked by pertussis toxin pretreatment, which inactivates the GTP-binding proteins G_i , G_k and G_o , we assume that SS activates the same GTP-binding protein mediating the effects of SS on an inwardly rectifying K^+ channel as that reported for locus coeruleus and certain peripheral neurons (Mihara et al. 1987; North et al. 1987). However, the M-current and its activation by SS is pertussis toxin insensitive, which suggests that if a G protein is involved in the M-current, it must be something other than G_k , G_i , or G_o . A similar conclusion was reached by Dutar and Nicoll (Dutar and Nicoll 1988), whose studies further suggest that inositol triphosphate may mediate the muscarinic closure of M-channels in hippocampal pyramidal neurons. Combining these bits of data for the M-channel and inward rectifying K^+ channel leads us to postulate several possible postreceptor sites for ethanol interaction (figure 3). It would appear from such considerations that ethanol could act at one of several sites between the receptors for ACh and SS and their ion channels, including relevant G proteins and the ion channels themselves.

Finally, concerning the GABA hypothesis of ethanol action, our laboratory has been unable to find electrophysiologic evidence in two brain regions, either in vivo or in vitro, to support a GABA-like or GABA-potentiating effect of ethanol (see also Palmer this volume). First, we do not see ethanol potentiating either GABAergic IPSPs or responses to exogenous GABA. Second, we do not see pronounced or consistent depression of spontaneous discharge or hyperpolarizations with exogenous ethanol. Rather, GABAergic IPSPs seem to be reduced, consistent with reports of ethanol-induced reduction of GABA release (Howerton and Collins 1984; Strong and Wood 1984).

Several early reports claimed to find electrophysiologic evidence for ethanol enhancement of GABA effects, but these efforts either involved unbelievably low, nontoxicating (Nesteros 1980) or supralethal (Davidoff 1973) ethanol doses or very indirect measures of GABA responsivity (Mereu and Gessa 1985). More recent intracellular studies generally have come up with negative findings. For example, Barker's group (Harrison et al. 1987) found no evidence for GABA-potentiating effects of ethanol in hippocampal cultures, and studies from the Weight group (Lovinger et al. 1989; White and Weight 1988) found no effect of ethanol (up to 100 mM) on GABA-induced

A. Inward Rectifier

B. M-Channel

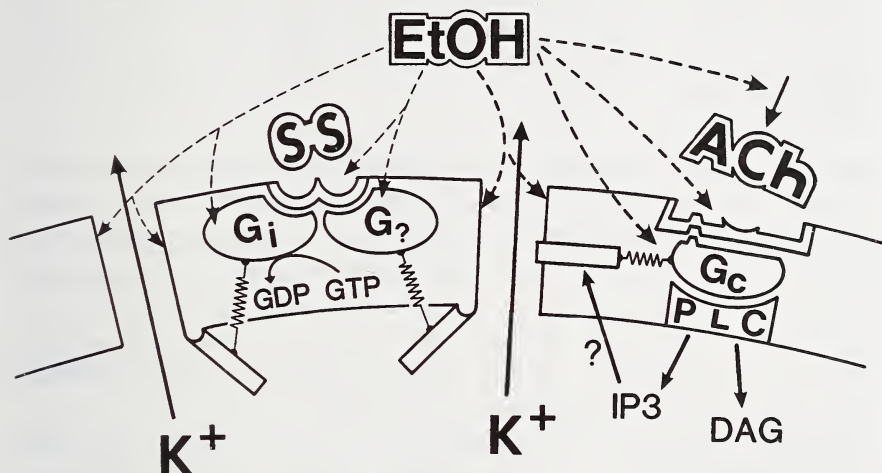


Figure 3.—Hypothetical loci of ethanol interaction with ACh and SS receptor-ionophore systems in hippocampal CA1 pyramidal neurons. SS may act on at least two K^+ channels in these cells: the inward rectifying, pertussis toxin (PTX)-sensitive channel and the PTX-insensitive, voltage-dependent M-channel. Dashed lines indicate possible sites of ethanol action, including the M-channel, reciprocally closed by ACh and opened by SS, and the inward rectifying channel that is opened by SS. Other possible sites of ethanol action include the SS receptor itself and/or possible GTP-binding proteins mediating the SS or ACh responses. $G_?$ refers to a possible non-PTX sensitive G protein; G_c alludes to a possible G protein (also PTX insensitive) that may be involved in the activation of phospholipase C (PLC) to produce the second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3) from membrane phospholipids. However, our data with atropine (see text) do not support an action of ethanol in releasing ACh or on the ACh muscarinic receptor (upper right).

Cl⁻ currents in acutely isolated dorsal root ganglia neurons or in hippocampal cultures. Locus coeruleus neurons in a slice preparation also show no effect of ethanol in GABA current (S. Schefner and Osmanovic, personal communication). In all of these studies, GABA responses were enhanced in the same cells by barbiturates and benzodiazepines; in the studies by the Weight group, ethanol in low concentrations (5-50 mM) antagonized NMDA responses (Lovinger et al. 1989).

To date there has been only one intracellular electrophysiologic study, on cultured spinal cord neurons (Celentano et al. 1988), that reports potentiation of GABA currents by ethanol. Even here there are problematic features, such as (1) the need for multiple short ethanol applications for ethanol to be effective, (2) the greater enhancement of glycine than GABA responses (not seen in biochemical studies), (3) no recovery from ethanol effects, (4) no controls for possible remote ("upstream") GABA or ethanol effects, and (5) no identification of the GABA current as to its Cl⁻ composition. Thus, it would seem that future electrophysiologic studies and the biochemical studies of Cl⁻ flux that support the ethanol/GABA hypothesis should emphasize controls that could provide possible explanations for this apparent discrepancy. One obvious problem might be that all of the biochemical studies have used preparations containing heterogeneous cell types, allowing for possible indirect effects of ethanol or GABA. For example, ethanol could be altering the release or action of transmitters (e.g., glutamate) or other substances that in turn enhance Cl⁻ flux, or ethanol could alter ionic cotransporters. Controls such as NA⁺ substitution or addition of Ca²⁺ channel antagonists or tetrodotoxin to block such release should be carried out in these studies. The contamination by other cell types of elements, such as glial or vascular tissue, in these biochemical preparations is also of some concern. The use of more homogeneous populations (e.g., "pure" glial or neuronal cultures) should be considered a priority. On the other hand, the mostly negative electrophysiologic data may indicate that the wrong neurons or recording conditions are being used and that other neuron types and/or media (e.g., containing steroids) should be assessed.

In the past, ethanol was thought to have relatively unspecific membrane actions. However, the studies described here and those from other

laboratories are beginning to point to a fairly specific group of effects of acutely administered ethanol. Thus, ethanol in intoxicating doses does not often appear to have much effect on the voltage-dependent conductances responsible for action potentials (but see Oakes and Pozos 1982) or on the passive conductances responsible for resting membrane properties. Rather, responses to neurotransmitters seem to display the highest initial sensitivity to ethanol, and some transmitter responses or mechanisms definitely appear more sensitive than others, depending on the cell type under study. Although ethanol will never be found to have only one action, we may ultimately find a small group of membrane processes that are sensitive at ethanol doses relevant to human intoxication. Considering the huge number of cellular activities and processes we could draw from, this could be a major step toward logical intervention or treatment.

References

- Adams, P.R.; Brown, D.A.; and Constanti, A.M. M-currents and other potassium current in bullfrog sympathetic neurons. *J Physiol* 330:537-572, 1982.
- Berger, T.; French, E.D.; Siggins, G.R.; and Bloom, F.E. Ethanol and some tetrahydroisoquinolines alter the discharge of rat hippocampal neurons in vivo when applied by microelectroosmosis or pressure: Relationship to opiate action. *Pharmacol Biochem Behav* 17:813-821, 1982.
- Bernardo, L.S., and Prince, D.A. Acetylcholine induced modulation of hippocampal pyramidal neurons. *Brain Res* 211:227-234, 1981.
- Berry, M.S., and Pentreath, V.W. The neurophysiology of alcohol. In: Sandler, M., ed. *Psychopharmacology of Alcohol* New York: Raven Press, 1980. pp. 43-72.
- Bloom, F.E., and Siggins, G.R. Electrophysiological action of ethanol at the cellular level. *Alcohol* 4:331-337, 1987.
- Bloom, F.E.; Siggins, G.R.; Foote, S.L.; Gruol, D.; Aston-Jones, G.; Rogers, J.; Pittman, Q.; and Staunton, D. Noradrenergic involvement in the

- cellular actions of ethanol. In: Usdin, E., ed. *Catecholamines, Neurology and Neurobiology*. Vol. 13. New York: Alan R. Liss, 1984. pp. 159-168.
- Brown, D.A., and Adams, P.R. Muscarinic suppression of a novel voltage-sensitive K^+ -current in a vertebrate neurone. *Nature* (London) 283:673-676, 1980.
- Carlen, P.L.; Gurevich, N.; and Durand, D. Ethanol in low doses augments calcium-mediated mechanisms measured intracellularly in hippocampal neurons. *Science* 215:306-309, 1982.
- Celentano, J.J.; Gibbs, T.T.; and Farb, D.H. Ethanol potentiates GABA- and glycine-induced chloride currents in chick spinal cord neurons. *Brain Res* 455:377-380, 1988.
- Davidoff, R.A. Alcohol and presynaptic inhibition in an isolated spinal cord preparation. *Arch Neurol* 28:60-63, 1973.
- Dodd, J.; Dingledine, R.; and Kelly, J.S. The excitatory action of acetylcholine on hippocampal neurones of the guinea pig and rat maintained in vitro. *Brain Res* 207:109-127, 1981.
- Dutar, P., and Nicoll, R.A. Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger systems: Electrophysiological studies in vitro. *J Neurosci* 8:4214-4224, 1988.
- Faber, D.S., and Klee, M.R. Actions of ethanol on neuronal membrane properties and synaptic transmission. In: Blum, K., ed. *Alcohol and Opiates: Neurochemical and Behavioral Mechanisms*. New York: Academic Press, 1977. pp. 41-63.
- Gahwiler, B.H., and Brown, D.A. Muscarine affects calcium-currents in rat hippocampal pyramidal cells in vitro. *Neurosci Lett* 76:301, 1987.
- Grenell, R.G. Effects of alcohol on the neuron. In: Kissin, B., and Begleiter, H., eds. *Biology of Alcoholism*. Vol. 1. New York: Plenum Press, 1982. pp. 1-19.

- Halliwel, J.V., and Adams, P.R. Voltage-clamp analysis of muscarinic excitation in hippocampal neurons. *Brain Res* 250:71- 92, 1982.
- Harrison, N.L.; Majwesky, M.D.; Harrington, J.W.; and Barker, J.L. Structure-activity relationships for steroid interaction with the gamma-aminobutyric acid_A receptor complex. *J Pharmacol Exp Ther* 241:346-353, 1987.
- Howerton, T.C., and Collins, A.C. Ethanol-induced inhibition of GABA release from LS and SS mouse brain slices. *Alcohol* 1:471- 477, 1984.
- Inoue, M.; Nakajima, S.; and Nakajima, Y. Somatostatin reduced excitability by enhancing the inward rectification in cultured locus coeruleus neurons. *Soc Neurosci Abstr* 13:1308, 1987.
- Jacquin, T.; Champagnat, J.; Madamba, S.; Denavit-Saubie, M.; and Siggins, G.R. Somatostatin depresses excitability in neurons of the solitary tract complex through hyperpolarization and augmentation of I_M , a noninactivating voltage-dependent outward current blocked by muscarinic agonists. *Proc Natl Acad Sci USA* 85:948-952, 1988.
- Kalant, H. Ethanol and the nervous system. Experimental neurophysiological aspects. *Int J Neurol* 9:111-124, 1974.
- Lovinger, D.M.; White, G.; and Weight, F.F. Ethanol inhibits NMDA activated ion currents in hippocampal neurons. *Science* 243:1721-1724, 1989.
- Luini, A.; Lewis, D.; Guild, S.; Schofield, G.; and Weight, F. Somatostatin, an inhibitor of ACTH secretion, decreases cytosolic free calcium and voltage-dependent calcium current in a pituitary cell line. *J Neurosci* 6:3128-3132, 1986.
- Madison, D.V.; Lancaster, B.; and Nicoll, R.A. Voltage clamp analysis of cholinergic action in the hippocampus. *J Neurosci* 7:733-741, 1987.

- Mancillas, J.R.; Siggins, G.R.; and Bloom, F.E. Ethanol selectively enhances responses to acetylcholine and somatostatin in the rat hippocampus. *Science* 231:161-163, 1986a.
- Mancillas, J.R.; Siggins, G.R.; and Bloom, F.E. Somatostatin selectively enhances acetylcholine-induced excitations in rat hippocampus and cortex. *Proc Natl Acad Sci USA* 83:7518-7521, 1986b.
- Mereu, G., and Gessa, G.L. Low doses of ethanol inhibit the firing of neurons in the substantia nigra, pars reticulata: A GABAergic effect? *Brain Res* 360:325-330, 1985.
- Mihara, S.; North, R.A.; and Surprenant, A.M. Somatostatin increases an inwardly rectifying potassium conductance in guinea-pig submucous plexus neurones. *J Physiol* 390:335-355, 1987.
- Moore, S.D.; Madamba, S.G.; Joels, M.; and Siggins, G.R. Somatostatin augments the M-current hippocampal neurons. *Science* 239:278-280, 1988.
- Nesteros, N.S. Ethanol specifically potentiates GABA-mediated neurotransmission in feline cerebral cortex. *Science* 209:708-710, 1980.
- North, R.A.; Williams, J.T.; Surprenant, A.M.; and Christie, M.J. Mu and delta receptors belong to a family of receptors that are coupled to potassium channels. *Proc Natl Acad Sci USA* 84:5487-5491, 1987.
- Oakes, S.G. and Pozos, R.S. Electrophysiologic effects to acute ethanol exposure. II. Alterations in the calcium component of action potentials from sensory neurons in dissociated culture. *Dev Brain Res* 5:251-255, 1982.
- Pittman, Q.J., and Siggins, G.R. Somatostatin hyperpolarizes hippocampal pyramidal cells in vitro. *Brain Res* 221:402-408, 1981.
- Rogers, J.; Siggins, G.R.; Schulman, J.A.; and Bloom, F.E. Physiological correlates of ethanol intoxication, tolerance, and dependence in rat cerebellar Purkinje cells. *Brain Res* 196:183-198, 1980.

- Rogers, J.; Madamba, S.G.; Staunton, D.A.; and Siggins, G.R. Ethanol increases single unit activity in the inferior olivary nucleus. *Brain Res* 385:253-262, 1986.
- Scharfman, H.E., and Schwartzkroin, P.A. Selective depression of GABA-mediated IPSPs by somatostatin in area CA1 of rabbit hippocampal slices. *Soc Neurosci Abstr* 14:115, 1988.
- Siggins, G.R., and Bloom, F.E. Alcohol-related electrophysiology. *Pharmacol Biochem Behav* 13:203-211, 1981.
- Siggins, G.R., and Gruol, D.L. Mechanisms of transmitter action in the vertebrate central nervous system. In: *Handbook of Physiology*. Volume on Intrinsic Regulatory Systems of the Brain, F.E. Bloom, ed. Bethesda, MD: The American Physiological Society, 1986. pp. 1-114.
- Siggins, G.R.; Bloom, F.E.; French, E.D.; Madamba, S.G.; Mancillas, J.; Pittman, Q.J.; and Rogers, J. Electrophysiology of ethanol on central neurons. *Ann NY Acad Sci* 49:350-366, 1987a.
- Siggins, G.R.; Pittman, Q.J.; and French, E.D. Effects of ethanol on CA1 and CA3 pyramidal cells in the hippocampal slice preparation: An intracellular study. *Brain Res* 414:22-34, 1987b.
- Strong, R., and Wood, W.G. Membrane properties and aging: In vivo and in vitro effects of ethanol on synaptosomal gamma-aminobutyric acid (GABA) release. *J Pharmacol Exp Ther* 229:726-730, 1984.
- Suzdak, P.; Glowa, J.R.; Crawley, J.N.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. A selective imidazodiazepine antagonist of ethanol in the rat. *Science* 234:1243-1247, 1986.
- Tsunoo, A.; Yoshii, M.; and Narahashi, T. Block of calcium channels by enkephalin and somatostatin in neuroblastoma-glioma hybrid NG 108-15 cells. *Proc Natl Acad Sci USA* 83:9832-9836, 1986.
- White, G., and Weight, F.F. Ethanol does not increase the amplitude of the GABA-activated Cl^- current in isolated dorsal root ganglion neurons of adult rats. *Abstr Int Symp Alcohol Seizures*, p. 24, 1988.

Yamashita, N.; Kojima, I.; Shibuya, N.; and Ogata, E. Pertussis toxin inhibits somatostatin-induced K^+ conductance in human pituitary tumor cells. *Am J Physiol* 253:E28-E32, 1987.

Discussion

HARRIS: In our paper in 1986 (*Life Sci* 39:2005), we could not show any effect of ethanol in hippocampus on Cl^- flux in either SS or LS mice, so that agrees perfectly with your comments. The second potential problem is using anesthetized animals. We have shown that other anesthetics act on the Cl^- channel and that if it is already maximally activated, no further effect will be seen. In the spinal cord, there is evidence that ethanol alone enhances the Cl^- flux. Thus, there may be some sort of diversity of chloride channels. I agree completely with George that there could be diversity in other factors. In defense of the Cl^- flux measurements, we put in radioactive chloride so we know what ion we are measuring, in contrast to electrophysiologic studies.

SIGGINS: Actually, in the voltage clamp studies by Weight and co-workers on dorsal root ganglia neurons and by Joefferly Barker (on hippocampal neurons), they have characterized the Cl^- currents too. Those same channels are enhanced by benzodiazepines and by pentobarbital in the same cell where it is not enhanced by ethanol. In those studies, they can alter chloride levels and alter the GABA effect according to the Nernst equation.

HARRIS: Maybe removal of Cl^- inactivates a Ca^{2+} channel or something like that.

SIGGINS: We could easily turn this argument around. In spinal cord, for example, none of those studies that you have mentioned used isolated systems. While isolated systems have been given a bum rap, one could argue both sides of the fence equally well. In the spinal cord studies you mentioned, nobody has attempted to block the remote effects of GABA, so it could be releasing or blocking the release of other agents, which then has the ultimate effect that you are seeing on Cl^- flux. Thus, we have different sets of problems in the two systems, and the next step is to do the kind of controls that each of us sees are needed in the other's preparation.

QUESTION: What is the situation with the spare receptors or spare channels?

SIGGINS: A major question is what percentage of the channels is required to cause a spike in the case of Na^+ channels, for example, and what fraction of the channels for Ca^{2+} carry the current in the later phases. A small ethanol-induced change might not be reflected in what we see in the spike. You might need to know out 90 percent of the channels to see a spike change. The same thing could be occurring with the GABA Cl^- flux. Finally, let me say one thing about isolation. To get a clear idea of the *primary* effect of ethanol, at some point you are going to have to isolate. Patch clamp has been criticized here, but patch clamp can be done in a situation where you are not losing or changing intracellular constituents, where you do not alter the factors which go into regulating cellular function. Ideally, we should be comparing results from isolated systems with intact systems and trying to get some cohesive data.

GOLDSTEIN: McClearn's crisp assertion that you can't do just one thing in a selective breeding program also applies to the use of drugs, even one as simple as ethanol. All compounds have a multitude of effects. On the other hand, you have to start someplace by looking at one effect and following it up.

PALMER: I would like to comment on something that Barry and George brought up and turn it into a question for George. That is one of dosage. There is a lot of argument about what an appropriate dose of ethanol is. In our studies of in oculo grafts of human tissue, some neurons can be depressed in the range of 3 mM, which is quite appropriate. Cerebellar grafts from LS mice are depressed at around 20-30 mM, whereas grafts from SS cerebellum require closer to 300-400 mM to achieve 50 percent depression of the firing rate of the Purkinje cells. Those concentrations are relevant to the brain concentrations in these mice when ethanol causes ataxia. Fisher 344 rats are very sensitive to ethanol, while the Marshall rats are relatively insensitive. Thus, the dosages used should be relevant to the amounts needed to bring about behavioral differences and not necessarily correlated with the doses required in humans.

Neuropeptides

Evidence for Site Selectivity of Thyrotropin-Releasing Hormone Interactions With Ethanol-Induced Central Nervous System Dysfunction¹

Hugh E. Criswell and George R. Breese²

Introduction

Thyrotropin-releasing hormone (TRH) was the first of the releasing factors isolated from the hypothalamus and synthesized (Guillemin 1978; Schally 1978). TRH is a tripeptide (pyroglutamyl-histidyl-prolineamide) named for its ability to release thyrotropin (TSH) from the pituitary. Subsequent investigations revealed that behavioral responses following L-DOPA administration to mice treated with pargyline were enhanced by TRH and that this augmentation of L-DOPA-induced behavior was also observed in hypophysectomized mice (Plotnikoff et al. 1972). This latter finding and localization of TRH in regions other than the hypothalamus (Brownstein et al. 1974; Jackson and Reichlin 1974) provided support for the view that TRH was influencing central functions unrelated to the hypothalamic-pituitary-thyroid axis (Plotnikoff et al. 1972; Breese et al. 1974).

¹This work was supported by grants NS-26595 and AA-08024.

²Center for Alcohol Studies and the Biological Sciences Research Center, School of Medicine, University of North Carolina, Chapel Hill, NC 27599.

Other observations also provided support for this view that TRH influenced central functions unrelated to its ability to release TSH. For example, TRH was found to antagonize the sedative action of pentobarbital in hypophysectomized mice (Prange et al. 1974; Breese et al. 1975). Because of the effectiveness of TRH against pentobarbital sedation, Breese et al. (1974) explored whether TRH would antagonize ethanol-induced sedation. The purpose of this paper is to review this early work concerning ethanol-TRH interactions and to describe how TRH has been used to localize a site in brain involved in the sedation that accompanies ethanol administration. This critique focusing specifically on TRH seems appropriate, as TRH was the first endogenous peptide demonstrated to have a profound effect on the pharmacology of ethanol (Breese et al. 1974).

Effects of TRH on the Sedation Induced by Ethanol

Administration of TRH was found to antagonize ethanol narcosis (sleep time) after oral as well as systemic administration (Breese et al. 1974; Cott et al. 1976). This analeptic action of TRH was proposed to be centrally mediated, as evidenced by the fact that microgram quantities of intraventricularly administered TRH were effective against ethanol sedation, compared with the greater doses required when TRH was administered systemically (Breese et al. 1974; Cott et al. 1976). The ratio of central to systemic TRH required for similar reductions in ethanol-induced sleep time is shown in table 1. Subsequent investigations of the distribution of TRH revealed that TRH immunoreactivity was elevated in the brain after systemic administration, demonstrating that TRH could enter the brain after peripheral administration (Mailman et al. 1979). Other evidence of the ability of TRH to reduce sedation includes its ability to antagonize the aerial righting deficit (Vogel et al. 1981) and the reduced locomotion induced by ethanol in rats (Breese et al. 1984a, 1985; Frye et al. 1983). This TRH antagonism of ethanol-induced sedation was found not to be species specific, as shown by the ability of TRH to antagonize ethanol in a variety of laboratory rodents (Cott et al. 1976). Reports that TRH would antagonize the sedation induced by pentobarbital (Prange et al. 1974; Breese et al. 1975) indicated that the analeptic action of TRH was not specific for ethanol. This view was reinforced by the finding that TRH would also antagonize the

Table 1.—Effects of central versus peripheral administration of TRH on ethanol-induced sleep in mice

Treatment	Dose	Mean sleep time + Standard error (percent of control)
Intraperitoneal	10 mg/kg	37.7±4.04
Intracisternal	43.4 ng/kg ^a	33.1±4.21

Central-to-peripheral ratio = $4.3 \times 10^6:1$.

^aMicroinjections of 1 μ g of TRH into a group of mice with an average weight of 19 g.

sedation produced by chlordiazepoxide, chloral hydrate, and reserpine (Prange et al. 1974; Breese et al. 1975).

Our early investigations documented that the TRH antagonism of ethanol was unrelated to an effect of TRH on ethanol metabolism or to metabolism of TRH. In this regard, ethanol levels were not altered by TRH, and mice were found to regain righting at higher blood ethanol concentrations after TRH administration when compared with untreated mice (Cott et al. 1976). Administration of the major metabolites of TRH as well as the amino acids found in this tripeptide were also found to be ineffective antagonists of ethanol-induced sedation. This latter finding indicated that metabolism of TRH was not responsible for its antagonism of ethanol-induced sedation (Breese et al. 1974; Cott et al. 1976).

Relationship of the TRH Analeptic Action of the Pituitary-Thyroid Axis

Several investigations divorced the analeptic action of TRH from an involvement of the pituitary-thyroid axis. For example, thyroid hormone (T3) was ineffective in reducing the sedation induced by ethanol, and hypophysectomy did not alter the TRH antagonism of ethanol-induced

sedation (Breese et al. 1974). The question raised by these results was whether the receptors responsible for this presumed extrahypothalamic action of TRH were like those found in the pituitary. After the isolation of TRH, several TRH analogs were synthesized and their ability to release TSH was defined. The compounds with TSH-releasing activity were also found to antagonize ethanol-induced sleep (Cott et al. 1976). These results suggested that the properties of the receptors in the pituitary were closely related to those in brain. Subsequent binding studies have supported this viewpoint (Taylor and Burt 1981, 1982; Sharif and Burt 1983).

Comparison of TRH With Other Endogenous Peptides

Another question asked during the course of our investigations was whether other endogenous peptides would influence ethanol sedation. This work demonstrated that none of the other known hypothalamic releasing factors influenced ethanol-induced sleep time (Cott et al. 1976). However, substance P was a peptide capable of reducing ethanol sedation. Subsequent studies demonstrated that other peptides, including β -endorphin and neurotensin, were capable of enhancing ethanol-induced sedation (Frye et al. 1981). Thus, it can be concluded that a nonspecific action of peptide administration into brain cannot explain the analeptic action of TRH.

Behavioral Specificity of TRH Antagonism of Ethanol Action

In addition to reversing ethanol-induced sleep time, TRH also reversed several other measures of ethanol action on the central nervous system (CNS). Ethanol hypothermia (Breese et al. 1974) and ethanol-induced depression of cerebellar cGMP (Mailman et al. 1979) were reversed by TRH. As mentioned earlier, ethanol-induced depression of the aerial righting reflex (Vogel et al. 1981) and locomotor activity in Sprague-Dawley rats were also reversed by TRH (Breese et al. 1984b).

However, TRH does not antagonize all effects that follow ethanol administration. Withdrawal from chronic ethanol exposure results in a

variety of symptoms. TRH does not precipitate these symptoms in rats acutely withdrawn from chronic ethanol treatment (Frye et al. 1981), which suggests that TRH does not antagonize the antiseizure action of ethanol. In support of this view, TRH recently was found not to antagonize the ethanol blockade of seizure spread induced by inferior collicular stimulation (T.J. McCown, personal communication). Another important action of ethanol is its anxiolytic or anticonflict action. It has been established that TRH will not antagonize the anticonflict action of ethanol; quite the contrary, TRH enhances this action of ethanol (Vogel et al. 1981). Table 2, which summarizes the effects of TRH on several actions of ethanol, clearly indicates that TRH does not block all of the effects of ethanol on the CNS.

Relationship of the TRH-induced Stimulant Action to the Analeptic Action

Investigations have demonstrated that TRH could increase motor activity (Wei et al. 1975; Miyamota and Magawa 1977). This finding raised the question of whether the stimulant property of TRH was responsible for its ability to antagonize ethanol-induced sedation. For this reason, Breese et al. (1974) compared the action of TRH on ethanol-induced sleep with the

Table 2.—Effects of TRH on behaviors elicited by ethanol

Behavior	Ethanol	TRH + Ethanol ^a	TRH alone
Sleep time	Increase	Decrease	No effect
Aerial righting	Increase	Decrease	No effect
Locomotion	Decrease	Increase	Increase
Seizure activity	Decrease	No effect	No effect
cGMP	Decrease	Increase	Increase
Conflict behavior	Decrease	Decrease	Decrease
Temperature	Decrease	Increase	Increase

^a"Decrease" indicates that TRH brought scores induced by ethanol significantly toward baseline; and "increase" means that TRH augmented the ethanol effect.

change produced by d-amphetamine. Whereas TRH produced its expected reduction of sleep time induced by ethanol, d-amphetamine enhanced ethanol-induced sleep time. Subsequently, Vogel et al. (1981) compared the effects of TRH, amphetamine, naloxone, and fenmetozol on ethanol impairment of the aerial righting reflex and anticonflict action. This investigation showed that d-amphetamine did not block ethanol-induced depression of the aerial righting reflex. In fact, d-amphetamine augmented this effect while blocking the anticonflict action of ethanol. This pattern of action against ethanol is opposite that of TRH on the loss of aerial righting and the anticonflict action of ethanol. Naloxone at a high dose of 20-60 mg/kg decreased ethanol action on both tasks, and only fenmetozol produced the same pattern of action as TRH. When individual behaviors were compared, TRH also produced a different pattern of behaviors than did amphetamine (Ervin et al. 1981). Thus, the action of TRH on ethanol sedation and anticonflict activity is not simply that of a central stimulant. This work emphasizes the point that even though a compound may antagonize one function of ethanol, other central actions may not be altered or may even be enhanced by these agents. The differing action of these ethanol antagonists suggests that ethanol may act by affecting more than one neurochemical system.

Relationships of TRH-induced Antagonism of Ethanol-induced Sedation to Transmitter Mechanisms

One question raised about the ability of TRH to antagonize ethanol-induced depression was whether this action could be related to classical transmitter mechanisms. An important observation in this regard was the fact that atropine was found to antagonize the analeptic action of TRH in mice, allowing us to conclude that a cholinergic link exists in the analeptic action of TRH (Cott et al. 1976). This conclusion has support from the electrophysiologic studies of Yarbrough (1983). It should be pointed out that cholinergic antagonists apparently do not antagonize the action of TRH to reduce sedation induced by ethanol in rats as they do in mice (Mailman et al. 1979; Santori et al. 1981).

Cott et al. (1977) proposed that TRH may interact with a γ -aminobutyric acid (GABA) mechanism. This view is also supported by the displacement of TRH from its binding site by benzodiazepines (Sharif and Burt 1984) and the opposing effects of TRH and the GABA agonist muscimol microinjected into the medial septum on ethanol sleep time (McCown et al. 1986a). Breese et al. (1984b) evaluated the possibility that TRH had a GABA antagonist action by comparing the effects of bicuculline and TRH at specific sites in brain. Because TRH did not induce convulsive behavior when placed in the inferior colliculus as did bicuculline when placed into this site, it was concluded that TRH was not acting as a "pure" GABA antagonist. It remains problematic whether TRH interacts with other sites on the GABA-benzodiazepine receptor complex.

Sites of Action of TRH: Relationship to the Central Actions of Ethanol

It is our philosophy that identification of brain regions important to a function impaired by ethanol should allow better resolution of potential neural mechanisms related to the actions of ethanol. The cytotoxic effects and rapid diffusion of ethanol have precluded its microinjection into specific brain areas as a viable approach to define sites at which ethanol alters CNS function. For this reason, we have used an alternative approach of giving ethanol systemically and microinjecting compounds that antagonize or enhance selected actions into various brain sites to identify a site where they influence an action of ethanol.

The effectiveness of TRH as an ethanol antagonist has provided a tool with which to examine where ethanol exerts its sedative action in brain. For example, by administering systemic ethanol and then microinjecting TRH into discrete brain areas, it should be possible to determine where in brain TRH can act to block a specific behavioral effect of ethanol. Subsequent work has shown that the medial septal nucleus is a major site for TRH antagonism of ethanol sedation (Breese et al. 1984b; McCown et al. 1985). Microinjection of 1 μ g of TRH into the medial septum reduced ethanol sleep time, whereas microinjections into the inferior colliculus, nucleus accumbens, raphe obscurus, or substantia nigra were ineffective (Breese et al. 1984b; McCown et al. 1985). Interestingly, TRH microinjection into the medial

septum does not antagonize all of the actions of ethanol. While microinjection of TRH into the medial septum reversed the effects of ethanol on sleep time and locomotor activity, the hypothermia induced by ethanol was not altered (Breese et al. 1984b). Intracisternal microinjection of TRH does block ethanol-induced hypothermia, which indicates that the effect is a central one but is not produced by an action on the medial septal nucleus. Furthermore, although TRH produces anticonflict activity when administered either systemically or intracisternally (Vogel et al. 1980), it is not effective in altering this action of ethanol when microinjected into the medial septum (unpublished observations). This evidence for site specificity of the action of TRH suggests that the sites of ethanol-induced hypothermia and anticonflict action are separate from the site mediating ethanol-induced sedation. It is known that TRH-induced changes in respiration can be elicited by microinjections into the raphe obscurus but not the medial septum (McCown et al. 1986a). Such evidence supports our contention that the neurobiologic basis of the central actions of ethanol can best be delineated at sites in brain that support the behavior under investigation.

Evidence for TRH Antagonism of Ethanol Alterations of Neural Activity

Peripheral administration of ethanol results in decreased neural activity in many brain areas (Klemm and Stevens 1974), but more recent studies have shown site dependency for ethanol effects on neural activity (Bloom and Siggins 1987). Givens and Breese (1988) showed that rhythmically bursting neurons in the medial septum were slowed in a dose-response fashion by ethanol. Pressure ejection of ethanol directly onto these neurons also produces a dose-related decrease in their activity (unpublished observations). When TRH is applied to medial septal neurons by iontophoresis, a majority of neurons are excited (Lamour et al. 1985). Iontophoretic application of TRH to rhythmic bursting neurons in the medial septum increases firing rate. A peripheral dose of ethanol sufficient to drastically decrease the activity of rhythmic bursting neurons does not affect this increase in cellular firing induced by TRH (unpublished observations). Thus, TRH appears to produce an excitatory effect on neurons in the medial septum that overcomes the inhibitory effect of ethanol on these same neurons. The fact that TRH does not excite neurons in all parts of brain (Winokur and Beckman 1978)

may explain the data showing that TRH does not antagonize all of the central deficits induced by ethanol. In brain areas where TRH and ethanol produce similar effects on neural activity, they also should produce similar effects on behavior.

Effect of TRH on Ethanol-induced and Other Sedative Drug-induced Behaviors in Humans

TRH appears to be a relatively safe drug in rodents, where toxicologic studies have shown the 50 percent lethal dose to be 2-4 g/kg (Piva and Steiner 1972). A great deal of information concerning the effect of TRH in humans has resulted from clinical trials of TRH for treatment of amyotrophic lateral sclerosis (ALS). The side effects of TRH noted in these studies include tremor, respiratory stimulation, mild hyperthermia, chills, and nausea. These effects first appear at the microgram level after systemic administration of TRH and continue through levels as high as 10 mg/kg (Gracco et al. 1984; Sufit et al. 1984). There has been surprisingly little adverse reaction to long-term administration of TRH in patients with ALS, although the continued discomfort associated with the stated undesirable effects of TRH has been a drawback (Brooke et al. 1986). Regardless, these trials emphasize the low toxicity of TRH.

To our knowledge, there are only two studies of TRH as an antagonist to depressant drugs. Linnoila et al. (1981) failed to block the intoxicating effects of 1.5 g/kg of ethanol in humans with 10 μ g/kg of TRH. In fact, their subjects subjectively reported an increase in intoxication. In contrast to this finding, Garbett et al. (in press) found that 50 μ g of TRH blocked the depressant effect of chlordiazepoxide in human volunteers. This ambiguity of findings likely results from the different measures taken, the different drugs, and the low dose of TRH administered in these studies.

There has been a recent revival of interest in drugs that may have the ability to antagonize ethanol-induced sedation. This interest was stimulated by the finding that Ro 15-4513, a drug acting on the benzodiazepine receptors, would reduce ethanol-induced sleep (Suzdak et al. 1986). This compound, however, possesses one major property that would prevent it from being used clinically: it is proconvulsant (Nutt and Lister 1987; unpublished data).

Therefore, such a compound would not be a useful treatment of ethanol overdose if withdrawal from ethanol would result in hyperirritability, as is often the case, because generalized seizures would likely result. The clinical trials in ALS patients have documented the relative safety of TRH. Animal studies have demonstrated the ability of TRH to antagonize ethanol sedation, to reduce ethanol lethality, and to increase respiration. Furthermore, TRH does not precipitate ethanol withdrawal or alter the antiseizure properties of ethanol and does not itself exacerbate withdrawal symptoms in animals (Frye et al. 1983). Therefore, until adequate doses of TRH are tested in human patients, the question of whether TRH would be a useful ethanol antagonist against acute ethanol overdose in a clinical setting remains problematic.

References

- Bloom, F.E., and Siggins, G.R. Electrophysiological action of ethanol at the cellular level. *Alcohol* 4:331-337, 1987.
- Breese, G.R.; Cott, J.M.; Cooper, B.R.; Prange, A.J.; and Lipton, M.A. Antagonism of ethanol narcosis by thyrotropin releasing hormone. *Life Sci* 14:1053-1063, 1974.
- Breese, G.R.; Cott, J.M.; Cooper, B.R.; Prange, A.J.; Lipton, M.A.; and Plotnikoff, N.P. Effects of thyrotropin-releasing hormones (TRH) on the actions of pentobarbital and other centrally acting drugs. *J Pharmacol Exp Ther* 193:11-22, 1975.
- Breese, G.R.; Coyle, S.; Frye, G.D.; and Mueller, R.A. Effects of TRH, ethanol, and TRH-ethanol combination on activity in rats with altered monoamine content. *Pharmacol Biochem Behav* 22:1013-1018, 1985.
- Breese, G.R.; Coyle, S.; Towle, A.C.; Mueller, R.A.; McCown, T.J.; and Frye, G.D. Ethanol-induced locomotor stimulation in rats following thyrotropin releasing hormone. *J Pharmacol Exp Ther* 229:731-737, 1984a.
- Breese, G.R.; Frye, G.D.; McCown, T.J.; and Mueller, R.A. Comparison of the CNS effects induced by TRH and bicuculline after microinjection into various brain sites: Absence of support for a GABA antagonist action for TRH. *Pharmacol Biochem Behav* 21:145-149, 1984b.

- Brooke, M.H.; Florence, J.M.; Heller, S.L.; Kaiser, K.K.; Phillips, D.; Gruber, A.; Babcock, D.; and Miller, J.P. Controlled trial of thyrotropin releasing hormone in amyotrophic lateral sclerosis. *Neurology* 36:146-151, 1986.
- Brownstein, M.J.; Palkovits, M.; Saavedra, J.M.; Bassir, R.M.; and Utiger, R.D. Thyrotropin-releasing hormone in specific nuclei of rat brain. *Science* 185:267-269, 1974.
- Cott, J.M.; Breese, G.R.; Cooper, B.R.; Barlow, T.S.; and Prange, A.J. Investigations into the mechanism of reduction of ethanol sleep by thyrotropin-releasing hormone. *J Pharmacol Exp Ther* 196:594-604, 1976.
- Cott, J.M., and Engel, J. Antagonism of the activity of thyrotropin releasing hormone by agents which enhance GABA transmission. *Psychopharmacology* 52:145-149, 1977.
- Ervin, G.N.; Frye, G.D.; Luttinger, D.; Nemeroff, C.B.; Vogel, R.A.; Prange, A.J.; and Breese, G.R. Modification of the actions of ethanol by centrally active peptides. *Peptides* 2(Suppl. 1):99-106, 1981.
- Frye, G.D.; McCown, T.J.; and Breese, G.R. Characterization of susceptibility of audiogenic seizures in ethanol-dependent rats after microinjection of alpha-amino acid (GABA) agonists into the inferior colliculus, substantia nigra, or medial septum. *J Pharmacol Exp Ther* 227:663-670, 1983.
- Garbett, J.C.; Gillette, G.M.; and Hicks, R.E. Thyrotropin releasing hormone modulates the neurobehavioral effects of chlordiazepoxide in man: Preliminary findings. *Proc Natl Acad Sci USA* (in press).
- Givens, B.S., and Breese, G.R. Ethanol suppresses activity of rhythmically bursting neurons of the medial septal area in freely moving rats. *Abstr Soc Neurosci* 14:193, 1988.
- Gracco, V.L.; Caligiuri, M.; Abbs, J.H.; Sufit, R.L.; and Brooks, B.R. Placebo controlled computerized dynametric measurements of bulbar and somatic muscle strength increase in patients with amyotrophic lateral

- sclerosis following intravenous infusion of 10 mg/kg thyrotropin-releasing hormone. *Ann Neurol* 16:110, 1984.
- Guillemin, R. Peptides in the brain: The new endocrinology of the neuron. *Science* 202:390-401, 1978.
- Jackson, I.M.D., and Reichlin, S. Thyrotropin-releasing hormone (TRH): Distribution in hypothalamic and extrahypothalamic brain tissue of mammalian and submammalian chordates. *Endocrinology* 95:854-862, 1974.
- Klemm, W.R., and Stevens, R.E. Alcohol effects on EEG and multiple-unit activity in various brain regions of rats. *Brain Res* 70:361-368, 1974.
- Lamour, Y.; Dutar, P.; and Jobert, A. Effects of TRH, cyclo-(His-Pro) and (3-Me-His²) TRH on identified septohippocampal neurons in the rat. *Brain Res* 331:343-347, 1985.
- Linnoila, M.; Mattila, M.J.; Karhunen, P.; Nuotto, E.; and Seppala, T. Failure of TRH and ORG 2766 hexapeptide to counteract alcoholic inebriation in man. *Eur J Clin Pharmacol* 21:27-32, 1981.
- Mailman, R.B.; Frye, G.D.; Mueller, R.A.; and Breese, G.R. Changes in brain guanosine-3', 5'-monophosphate (cGMP) content by thyrotropin-releasing hormone. *J Pharmacol Exp Ther* 208:169-175, 1979.
- McCown, T.J.; Frye, G.D.; and Breese, G.R. Evidence for site specific ethanol actions in the CNS. *Alcohol Drug Res* 6:423-429, 1985.
- McCown, T.J.; Hedner, J.A.; Towle, A.C.; Breese, G.R.; and Mueller, R.A. Brainstem localization of a thyrotropin-releasing hormone-induced change in respiratory function. *Brain Res* 373:189-196, 1986a.
- McCown, T.J.; Moray, L.J.; Kizer, J.S.; and Breese, G.R. Interactions between TRH and ethanol in the medial septum. *Pharmacol Biochem Behav* 24:1269-1274, 1986b.

- Miyamota, M., and Nagawa, Y. Mesolimbic involvement in the locomotor stimulant action of thyrotropin-releasing hormone (TRH) in rats. *Eur J Pharmacol* 44:143, 1977.
- Nutt, D.J., and Lister, R.G. The effect of the imidazodiazepine Ro 15-4513 on the anticonvulsant effects of diazepam, sodium pentobarbital and ethanol. *Brain Res* 413:193-196, 1987.
- Piva, F., and Steiner, H. Bioassay and toxicology of TRH. *Frontier Hormone Res* 1:11-21, 1972.
- Plotnikoff, N.P.; Prange, A.J.; Breese, G.R.; Anderson, M.S.; and Wilson, I.D. Enhancement of DOPA activity by a hypothalamic hormone. *Science* 178:417-418, 1972.
- Prange, A.J.; Breese, G.R.; Cott, J.M.; Martin, B.R.; Cooper, B.R.; Wilson, I.C.; and Plotnikoff, N.P. Thyrotropin releasing hormone: Antagonism of pentobarbital in rodents. *Life Sci* 14:447-455, 1974.
- Santori, E.M.; Schmidt, D.E.; Kalivas, P.W.; and Horita, A. Failure of muscarinic blockade to antagonize analepsis induced by thyrotropin-releasing hormone and MK-771 in the rat. *Psychopharmacology* 74:13-16, 1981.
- Schally, A.V. Aspects of hypothalamic regulation of the pituitary gland. *Science* 202:18-28, 1978.
- Sharif, N.A., and Burt, D.R. Biochemical similarity of rat pituitary and CNS TRH receptors. *Neurosci Lett* 39:57-63, 1983.
- Sharif, N.A., and Burt, D.R. Modulation of receptors for TRH by benzodiazepines: Brain regional differences. *J Neurochem* 43:742-746, 1984.
- Sufit, R.; Beaulieu, D.; Sanguq, M.; Paulson, D.; Reddan, W.; Erickson, L.; Montgomery, W.; Braun, S.; Peters, H.; and Brook, B.R. Placebo controlled quantitative measurements of neuromuscular function following intravenous infusion of 10 mg/kg thyrotropin-releasing hormone in 16

Neuropeptides

- male patients with amyotrophic lateral sclerosis. *Ann Neurol* 16:110-111, 1984.
- Suzdak, P.D.; Glowa, J.R.; Crawley, J.N.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. A selective imidazobenzodiazepine antagonist of ethanol in the rat. *Science* 243:1243-1247, 1986.
- Taylor, R.L., and Burt, D.R. Preparation of ^3H -[3-Me-His 2]TRH as an improved ligand for TRH receptors. *Neuroendocrinology* 32:310-316, 1981.
- Taylor, R.L., and Burt, D.R. Species differences in the brain regional distribution of receptor binding for thyrotropin-releasing hormone. *J Neurochem* 38:1649-1656, 1982.
- Vogel, R.A.; Frye, G.D.; Mann Koepke, K.; Mailman, R.B.; Mueller, R.A.; and Breese, G.R. Differential effects of TRH, amphetamine, naloxone, and fenmetozole on ethanol actions: Attenuation of the effects of punishment and impairment of aerial righting reflex. *Alcohol Clin Exp Res* 5:386-392, 1981.
- Vogel, R.A.; Frye, G.D.; Wilson, J.H.; Kuhn, C.M.; Mailman, R.B.; Mueller, R.A.; and Breese, G.R. Attenuation of the effect of punishment by thyrotropin-releasing hormone: Comparisons with chlordiazepoxide. *J Pharmacol Exp Ther* 212:153-161, 1980.
- Wei, E.; Siegel, S.; Loh, H.; and Way, E.L. Thyrotropin-releasing hormone and shaking behavior in rat. *Nature* (London) 253:279, 1975.
- Winokur, A., and Beckman, A.L. Effects of thyrotropin releasing hormone, norepinephrine and acetylcholine on the activity of neurons in the hypothalamus, septum and cerebral cortex of the rat. *Brain Res* 150:205-209, 1978.
- Yarbrough, G.G. Thyrotropin releasing hormone and CNS cholinergic neurons. *Life Sci* 33:111-118, 1983.

Ethanol and Opiate Receptors

*Paula L. Hoffman and Boris Tabakoff*¹

The "membrane hypothesis," used to explain the mechanism of action of ethanol at the molecular and cellular level, has been widely accepted and fruitful with respect to generating experiments that have advanced the understanding of the effects of ethanol in the central nervous system (CNS) and various organs (Tabakoff and Hoffman 1987). According to this hypothesis, many of the effects of ethanol result from the ability of the drug to partition into cell membranes and perturb the structure of the membrane lipids. A necessary corollary of this hypothesis, however, is that changes in cell membrane fluidity alter the properties of membrane-bound proteins (e.g., enzymes and receptors) that are the functional moieties of the cell membrane. Alternatively, ethanol could interact directly with hydrophobic portions (Lefkowitz and Caron 1988) of membrane-bound proteins, including neurotransmitter or hormone receptors. In the latter two instances, one can envision more specific sites of action for ethanol (i.e., "receptive areas" of particular proteins of lipid-protein interfaces [Tabakoff and Hoffman 1987]) than might be predicted from the relatively nonspecific lipid-ethanol interactions suggested by the membrane hypothesis.

The opiate receptor system has attracted considerable attention with regard to its possible susceptibility to perturbation by ethanol. A change in the binding of endogenous opiates produced by ethanol could conceivably play a role in the reinforcing or euphoriant effects as well as the addicting properties of ethanol. Several subtypes of opiate receptors have been described (Iwamoto and Martin 1981), and it has been found that in vitro

¹Division of Intramural Clinical and Biological Research, National Institute on Alcohol Abuse and Alcoholism, Rockville, MD 20852.

ethanol has selective effects on ligand binding to these receptor subtypes. In mouse striatal tissue, ethanol had a biphasic effect on dihydromorphine (DHM) binding to mu opiate receptors: a low concentration of ethanol (50 mM) increased binding, whereas higher concentrations inhibited binding (Tabakoff and Hoffman 1983). Ethanol inhibited only the binding of [2-D-Ala, 5-D-Leu]enkephalin (ENK) to striatal delta opiate receptors (Tabakoff and Hoffman 1983). The inhibitory effect of ethanol on ligand binding to both receptor subtypes resulted from alterations in the affinity of the receptors for the ligands (Tabakoff and Hoffman 1983). The striatal delta receptors were more sensitive than the mu receptors to the inhibitory effect of ethanol, as was also found in whole rat brain (Tabakoff and Hoffman 1983; Hiller et al. 1981). In mouse frontal cortex, however, ethanol had an approximately equal inhibitory effect on ligand binding to mu and delta opiate receptors, whereas binding to kappa receptors was unaffected by ethanol (Khatami et al. 1987).

In the studies described, the inhibitory effects of ethanol were observed at high ethanol concentrations (50 percent inhibitory concentrations ranging from 450 to 950 mM at 25 °C) (Tabakoff and Hoffman 1983; Khatami et al. 1987). These ethanol concentrations (equivalent to 2,070-4,370 mg percent) could not be attained in vivo, and these observed effects of ethanol on opiate binding could therefore not contribute to in vivo, behavioral effects of ethanol. Since relatively high concentrations of ethanol are also necessary to produce significant "fluidization" of bulk membrane lipids (Chin and Goldstein 1977), these inhibitory effects of high ethanol concentrations on opiate receptor binding might well be attributable to lipid perturbation, particularly considering the important role of lipids in opiate receptor function (Craves et al. 1979). This suggestion is supported by the finding that other short-chain alcohols alter ligand binding to opiate receptors in a manner similar to that of ethanol and that the potency of these alcohols is proportional to their membrane-water partition coefficients (Tabakoff and Hoffman 1983). Furthermore, we found that increasing assay temperature, which, like ethanol, is expected to increase membrane fluidity, reduced the affinity of striatal mu receptors for DHM, although ENK receptors were less affected (Hoffman et al. 1984). Thus, the differential sensitivity to ethanol of opiate receptor subtypes (Hoffman and Tabakoff 1986; Hiller et al. 1981; Khatami et al. 1987) and the different relative sensitivities of these receptor

subtypes in different brain areas (Hoffman and Tabakoff 1986; Khatami et al. 1987) may reflect variations in the lipid microenvironments of the receptors.

These data indicate that opiate receptors per se, like a number of other neurotransmitter and neuromodulator receptors, are little affected by concentrations of ethanol that are physiologically relevant (i.e., 10-50 mM). However, recent studies have indicated that the function of the guanine nucleotide-binding proteins (G proteins) that couple various receptors to their biochemical effectors is altered by low concentrations of ethanol. There is a family of G proteins, all of which have a similar, heterotrimeric structure (Weiss et al. 1988; Spiegel 1988). Each G protein has a distinct α subunit (α_s , α_i , etc.) that is crucial for its function, whereas the β/γ subunits are more similar among G proteins (Weiss et al. 1988; Spiegel 1988). Two proteins that mediate the coupling of receptors to adenylate cyclase are G_s and G_i ; the former is involved in stimulation of adenylate cyclase by agonists acting at stimulatory receptors, and the latter is involved in adenylate cyclase inhibition. At least three forms of G_i have been identified (Weiss et al. 1988; Spiegel 1988), as well as another protein, called G_o , which is present in high abundance in brain but the function of which has not yet been definitively determined. G proteins also couple receptors to membrane-bound phospholipase C and can directly affect the function of ion channels (Weiss et al. 1988; Spiegel 1988). For example, it has been suggested that G_{13} may be the protein that affects the function of K^+ channels (Codina et al. 1988) and that G_s can modulate the function of voltage-sensitive Ca^{2+} channels (Yatani et al. 1988).

The function of G proteins has been described in most detail for G_s , and in particular for the coupling of β -adrenergic receptors to adenylate cyclase (Weiss et al. 1988, Spiegel 1988). According to this model, interaction of agonist with the receptor leads to formation of a ternary complex consisting of agonist, receptor, and G protein. This complex has high affinity for agonist. The formation of the complex promotes release of GDP from α_s , and binding of GTP to α_s . The binding of GTP activates G_s , causing dissociation of the α_s and β/γ subunits, as well as dissociation of the receptor from the complex, and the return of the receptor to a conformation with low affinity for agonist. The α_s subunit, with GTP bound, associates with and

activates adenylate cyclase. A GTPase present within α_s hydrolyzes GTP to GDP, and G_s returns to its trimeric form, ending the cycle. The consequence of this cycle, in terms of ligand binding, is that in the absence of added guanine nucleotides, binding of agonists to β -adrenergic (or other) receptors is characterized by high- and low-affinity binding sites, representing, respectively, the agonist-receptor-G protein ternary complex and the receptor per se. In the presence of GTP or a nonhydrolyzable GTP analog, only low-affinity binding can be detected.

Ligand binding to opiate receptors is altered by guanine nucleotides, which suggests that G proteins are involved in the coupling of opiate receptors to their effectors (Childers and Snyder 1980; Chang et al. 1981; Pfeiffer et al. 1982). In addition, sodium ions are required for some actions of opiates and also influence opiate binding to receptors (Pert and Snyder 1974; Blume et al. 1979). However, the effects of GTP and Na^+ on opiate binding are additive, which suggests that these factors act at different sites (Childers and Snyder 1980). In mouse striatum, we found nonlinear Scatchard plots for [3H]DHM binding to mu receptors, which suggests the presence of high- and low-affinity binding sites. Both GTP and Na^+ reduced the affinity of the high-affinity binding sites for DHM (Hoffman et al. 1984). In the presence of GTP, the proportion of high-affinity DHM binding sites was also reduced, and the proportion of low-affinity sites was increased. In the presence of Na^+ , the number of high-affinity DHM binding sites was significantly decreased, and low-affinity sites were no longer detectable with the assay used (table 1). These data are consistent with the possibility that both coupling factors decrease the proportion of high-affinity binding sites and increase the proportion of low-affinity binding sites. It is difficult to measure low-affinity DHM binding with the direct filtration binding assay that was used in our studies; however, an increased number of low-affinity binding sites may also be reflected in the seemingly lower affinity for DHM of the striatal high-affinity mu receptors in the presence of GTP or Na^+ . We found similar effects of GTP and sodium ion on ENK binding to striatal delta receptors (i.e., lower affinity for agonist and lower number of high-affinity binding sites), although no low-affinity binding could be measured under the conditions of the assay (table 1).

The effects of low concentrations of ethanol on agonist binding to β -adrenergic receptors in mouse cortex, as well as on the stimulation of striatal

Table 1.—Effect of GTP and sodium ion on DHM and ENK binding to mouse striatal tissue^a

Measurement	DHM			ENK		
	Basal	GTP	Na ⁺	Basal	GTP	Na ⁺
K _H (nM)	0.6±0.1	1.3	1.2±0.2 ^b	1.0	1.3	1.7
R _H (fmol/mg of protein)	95±11	85.0	57±4 ^b	189.0	151.0	93.0
K _L (nM)	3.4±0.9	3.4				
R _L (fmol/mg of protein)	157±24	167.0				

^aTissue was prepared and DHM and ENK binding was assayed as described previously (Hoffman et al. 1982, 1984) in the absence (basal) or presence of 0.1 mM GTP (DHM), 1 mM GTP (ENK), or 100 mM NaCl. Values represent mean or mean ± standard error. Some data are taken from Hoffman et al. 1982.

^b*P* < 0.05 compared with basal value.

and cortical adenylate cyclase activity, have been suggested to be mediated primarily by an action on G_s (Valverius et al. 1987; Saito et al. 1985; Luthin and Tabakoff 1984). Furthermore, the effects of ethanol on phosphatidylinositol turnover in liver require the presence of a G protein (Rubin and Hoek 1988). Thus, G proteins appear to represent a sensitive and specific site(s) of action of ethanol that could lead to selective influences on receptor-effector coupling processes in vivo.

To assess the interaction of ethanol and G proteins with respect to opiate binding, the effect of a low concentration of ethanol on DHM and ENK binding to striatal receptors was assessed in the presence and absence of GTP or Na⁺ (figure 1). These studies were performed at 37 °C in vitro to more closely approximate in vivo conditions (Hoffman et al. 1984). While 25 mM ethanol alone had no significant effect on striatal DHM binding, the effect of ethanol was potentiated in the presence of GTP or Na⁺ such that there was a substantial reduction in DHM binding. In contrast, the effect of ethanol on ENK binding to delta receptors was not significantly altered by GTP or Na⁺ (figure 1). These data suggested that ethanol may influence

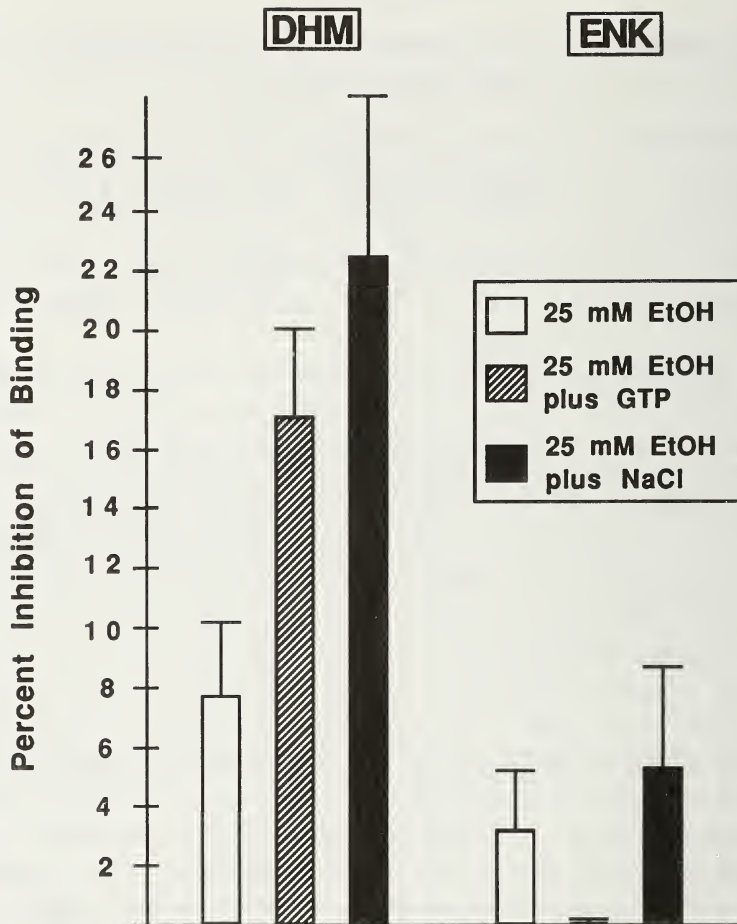


Figure 1.—Effect of ethanol on ligand binding to opiate receptors in the presence of Na^+ or GTP. $[^3\text{H}]\text{DHM}$ (0.2 nM) or $[^3\text{H}]\text{ENK}$ (.05 nM) binding to mouse striatal tissue was assayed as described previously (Hoffman et al. 1984). Binding was measured in the absence and presence of 25 mM ethanol, 0.1 mM GTP (DHM), 1 mM GTP (ENK), or 100 mM NaCl. Data are expressed as percent inhibition of binding, which was calculated by comparing the amount of DHM or ENK bound in the presence of ethanol alone with the amount bound in the absence of any additions, or the amount bound in the presence of ethanol plus GTP or NaCl with the amount bound in the presence of GTP or NaCl only. GTP alone produced approximately 50 percent inhibition of DHM or ENK binding and 75 percent inhibition of DHM binding. (From Hoffman et al. 1984.)

DHM binding to mu opiate receptors via an action at a G protein and that, by this mechanism, ethanol could have a significant effect on mu opiate receptor function at concentrations that can easily be attained in vivo (Kalant 1971).

Studies of chronic ethanol ingestion also support the postulate that mu opiate receptors are affected by physiologically relevant concentrations of ethanol. Administration of morphine to mice produces an increase in striatal dopamine synthesis (Urwyler and Tabakoff 1981), and after chronic ethanol ingestion, this response to morphine is disrupted (Hoffman et al. 1982). In addition, the affinity of striatal mu receptors for DHM is reduced in the mice fed ethanol chronically, as is the effect of the coupling factor, Na^+ , on DHM binding (Hoffman et al. 1982). Chronic ingestion of ethanol also results in a decrease in the number of high-affinity mu receptors in the mouse frontal cortex (Khatami et al. 1987). The data are consistent with an effect of chronic ethanol ingestion on mu receptor-effector coupling processes, leading to an uncoupling of the receptor and effector. Such a change would be similar to that seen in cerebral cortex of ethanol-fed mice, in which β -adrenergic receptors are uncoupled from adenylate cyclase (Saito et al. 1987). With respect to the β -adrenergic receptor system, the change involves an alteration in the quantity or function of G_s (Tabakoff et al. 1987).

In contrast to the results with mu receptors, ethanol in vivo would be expected to have little or no effect on ENK binding or function, since only high in vitro concentrations of ethanol inhibited ENK binding, and the effect of ethanol on ENK binding was not increased in the presence of GTP or Na^+ . In striatum, we (Hoffman et al. 1983) and others (Law et al. 1981) have demonstrated that delta opiate receptors are coupled to adenylate cyclase in an inhibitory manner. GTP and Na^+ are necessary for a demonstration of opiate inhibition of striatal adenylate cyclase, consistent with a role for G_i in this system (Blume et al. 1979). As predicted on the basis of the lack of effect of low concentrations of ethanol on ENK binding in the presence of GTP or Na^+ , ethanol in vitro had no effect on enkephalin-induced inhibition of mouse striatal adenylate cyclase (Hoffman and Tabakoff 1986). Ethanol in vitro also had no effect on cholinergic agonist-induced inhibition of adenylate cyclase in brain, which is also mediated by G_i (Hoffman et al. 1987; Rabin 1985). Furthermore, chronic ethanol ingestion by mice did not alter opiate

inhibition of striatal adenylate cyclase (Hoffman and Tabakoff 1986). These results suggest that ethanol, either acutely or chronically, has little effect on the function of the G_i protein that mediates inhibition of adenylate cyclase by enkephalin (possibly G_{i1} or G_{i2} ; Codina et al. 1988; Neer and Clapham 1988).

Overall, the data suggest that ethanol can selectively affect mu opiate receptor-G protein coupling processes. The immediate biochemical effector to which striatal or cortical mu receptors are coupled as well as the identity of the G protein involved in this coupling are not clear at present. Mu receptors have been suggested to be coupled to potassium channels (North et al. 1987), and in NG 108-15 cells in culture, opiate receptors were suggested to be coupled to calcium channels (Hescheler et al. 1987). As discussed above, G_{i3} has been suggested to be the G protein that influences the activity of potassium channels (Codina et al. 1988), whereas G_s has been suggested to affect voltage-dependent calcium channel activity (Yatani et al. 1988). However, it was suggested that G_o is the protein that couples opiate receptors to calcium channels in NG 108-15 cells (Hescheler et al. 1987). If the suggestion that mu opiate receptors are coupled to ion channels via a particular G protein is confirmed, and if such coupling also occurs in brain, our data suggest that these proteins may well represent a specific site at which ethanol can act to influence mu receptor function.

Whether the effects of ethanol are mediated by influences in lipid-protein interactions or by direct effects on proteins remains to be determined. However, because the biochemical effects of ethanol are selective, one may soon be able to focus on particular "receptive" proteins as the molecular site of action of ethanol. The rapid progress in determining the structure of membrane-bound receptors and G proteins (Lefkowitz and Caron 1988) and the ability to evaluate conformational changes in these proteins (e.g., Higashijima et al. 1987) promise to provide more detailed information on the site(s) and mechanism(s) of action of ethanol in the CNS. Such data will inevitably enhance our understanding of the processes by which ethanol can produce intoxication, reinforcement, tolerance, and physical dependence.

References

- Blume, A.J.; Lichtshtein, D.; and Boone G. Coupling of opiate receptors to adenylate cyclase: Requirements for Na^+ and GTP. *Proc Natl Acad Sci USA* 76:5626-5630, 1979.
- Chang, K.-J.; Hazum, E.; Killiam, A.; and Cuatrecasas, P. Interaction of ligands with morphine and enkephalin receptors are differentially affected by guanine nucleotide. *Mol Pharmacol* 20:1-7, 1981.
- Childers, S.R., and Snyder, S.H. Differential regulation by guanine nucleotides of opiate agonist and antagonist receptor interactions. *J Neurochem* 34:583-593, 1980.
- Chin, J.H., and Goldstein, D.B. Drug tolerance in biomembranes: A spin-label study of the effects of ethanol. *Science* 196:684-685, 1977.
- Codina, J.; Olate, J.; Abramowitz, J.; Mattera, R.; Cook, R.G.; and Birnbaumer, L. α_1-3 cDNA encodes that α subunit of G_k , the stimulatory G protein of receptor-regulated K^+ channels. *J Biol Chem* 263:6746-6750, 1988.
- Craves, F.B.; Zalc, B.; Leybin, L.; Baumann, N.; and Loh, H.H. Antibodies to cerebroside sulfate inhibit the effects of morphine and β -endorphin. *Science* 207:75-76, 1979.
- Hescheler, J.; Rosenthal, W.; Trautwein, W.; and Schultz, G. The GTP-binding protein, G_o , regulates neuronal calcium channels. *Nature (London)* 325:445-446, 1987.
- Higashijima, T.; Ferguson, K.; Sternweis, P.C.; Ross, E.M.; Smigel, M.D.; and Gilman, A.G. The effect of activating ligands on the intrinsic fluorescence of guanine nucleotide binding proteins. *J Biol Chem* 262:752-756, 1987.
- Hiller, J.M.; Angel, J.M.; and Simon, E.J. Characterization of the selective inhibition of the delta subclass of opioid binding sites by alcohols. *Science* 214:468-469, 1981.

- Hoffman, P.L.; Chung, C.T.; and Tabakoff, B. Effects of ethanol, temperature, and endogenous regulatory factors on the characteristics of striatal opiate receptors. *J Neurochem* 43:1003-1010, 1984.
- Hoffman, P.L.; Luthin, G.R.; Theodoropoulos, D.; Cordopatis, P.; and Tabakoff, B. Ethanol effects on striatal dopamine receptor-coupled adenylate cyclase and on striatal opiate receptors. *Pharmacol Biochem Behav* 18(1):355-359, 1983.
- Hoffman, P.L.; Saito, T.; and Tabakoff, B. Selective effects of ethanol on neurotransmitter receptor-effector coupling systems in different brain areas. *Ann NY Acad Sci* 492:396-397, 1987.
- Hoffman, P.L., and Tabakoff, B. Ethanol does not modify opiate-mediated inhibition of striatal adenylate cyclase. *J Neurochem* 46:812-816, 1986.
- Hoffman, P.L.; Urwyler, S.; and Tabakoff, B. Alterations in opiate receptor function after chronic ethanol exposure. *J Pharmacol Exp Ther* 222:182-189, 1982.
- Iwamoto, E.T., and Martin, N.R. Multiple opioid receptors. *Med Res Rev* 1:411-440, 1981.
- Kalant, H. Absorption, diffusion, distribution, and elimination of ethanol: Effects of biological membranes. In: Kissin, B., and Begleiter, H., eds. *The Biology of Alcoholism*. Vol. I. New York: Plenum Press, 1971. pp. 1-62.
- Khatami, S.; Hoffman, P.L.; Shibuya, T.; and Salafsky, B. Selective effects of ethanol on opiate receptor subtypes in brain. *Neuropharmacology* 26:1503-1507, 1987.
- Law, P.Y.; Wu, J.; Koehler, J.E.; and Loh, H.H. Demonstration and characterization of opiate inhibition of the striatal adenylate cyclase. *J Neurochem* 36:1834-1846, 1981.
- Lefkowitz, R.J., and Caron, M.G. Adrenergic receptors: Models for the study of receptors coupled to guanine nucleotide regulatory proteins. *J Biol Chem* 263:4993-4996, 1988.

- Luthin, G.R., and Tabakoff, B. Activation of adenylate cyclase by alcohols requires the nucleotide-binding protein. *J Pharmacol Exp Ther* 288:579-587, 1984.
- Neer, E.J., and Clapham, D.E. Roles of G protein subunits in transmembrane signalling. *Nature* (London) 333:129-134, 1988.
- North, R.A.; Williams, J.T.; Surprenant, A.; and Christie, J.M. μ and δ receptors belong to a family of receptors that are coupled to potassium channels. *Proc Natl Acad Sci USA* 84:5487- 5491, 1987.
- Pert, C.B., and Snyder, S.H. Opiate receptor binding of agonists and antagonists affected differentially by sodium. *Mol Pharmacol* 10:868-879, 1974.
- Pfeiffer, A.; Sadée, W.; and Herz, A. Differential regulation of the μ -, δ - and κ -opiate receptor subtypes by guanyl nucleotides and metal ions. *J Neurosci* 7:912-917, 1982.
- Rabin, R.A. Effect of ethanol on inhibition of striatal adenylate cyclase activity. *Biochem Pharmacol* 34:4329-4331, 1985.
- Rubin, R., and Hoek, J.B. Alcohol-stimulation of phospholipase C in human platelets requires G-protein activation. *Biochem J* 254:147-153, 1988.
- Saito, T.; Lee, J.M.; Hoffman, P.L.; and Tabakoff, B. Effects of chronic ethanol treatment on the β -adrenergic receptor-coupled adenylate cyclase system of mouse cerebral cortex. *J Neurochem* 48:1817-1822, 1987.
- Saito, R.; Lee, J.M.; and Tabakoff, B. Ethanol's effects on cortical adenylate cyclase activity. *J Neurochem* 44:1037-1044, 1985.
- Spiegel, A.M. G-proteins as receptor-effector couplers. *Ann Rev Med Chem* 23:235-242, 1988.
- Tabakoff, B., and Hoffman, P.L. Alcohol interactions with brain opiate receptors. *Life Sci* 32:197-204, 1983.

- Tabakoff, B., and Hoffman, P.L. Biochemical pharmacology of alcohol. In: Meltzer, H.Y., ed. *Psychopharmacology: The Third Generation of Progress*. New York: Raven Press, 1987. pp. 1521-1526.
- Tabakoff, B.; Valverius, P.; Nhamburo, P.T.; Rius, R.A.; and Hoffman, P.L. Region-specific effects of ethanol on G proteins in mouse brain. *Trans Am Soc Neurochem* 19:144, 1987.
- Urwyler, S., and Tabakoff, B. Stimulation of dopamine synthesis and release by morphine and D-Ala²-D-Leu⁵-enkephalin in the mouse striatum in vivo. *Life Sci* 28:2277-2286, 1981.
- Valverius, P.; Hoffman, P.L.; and Tabakoff, B. Effect of ethanol on mouse cerebral cortical β -adrenergic receptors. *Mol Pharmacol* 32:217-222, 1987.
- Weiss, E.R.; Kelleher, D.J.; Woon, C.W.; Soparkar, S.; Osawa, S.; Heasley, L.E.; and Johnson, G.L. Receptor activation of G protein. *FASEB J* 2:2841-2848, 1988.
- Yatani, A.; Imoto, Y.; Codina, J.; Hamilton, S.L.; Brown, A.M.; and Birnbaumer, L. The stimulatory G protein of adenylyl cyclase, G_s, also stimulates dihydropyridine-sensitive Ca²⁺ channels. *J Biol Chem* 263:9887-9895, 1988.

Discussion

SIGGINS: You mentioned that opiate receptors may be coupled to Ca²⁺ channels. I thought that the evidence was strongest for coupling to K⁺ channels.

HOFFMAN: Most of the evidence that I have seen is for coupling to K⁺ channels. However, there are some data from NG 108-15 cells suggesting coupling of opiate receptors to a Ca²⁺ channel.

Purification, cDNA Cloning, and Characterization of an Opioid-Binding Protein with Homologies to the Immunoglobulin Superfamily¹

Andrew P. Smith, Horace H. Loh, and Nancy M. Lee²

Introduction

The opioids are a highly diverse group of drugs, including both plant-derived alkaloids and a large number of peptides in the mammalian brain. In addition to their best known and clinically most important property, analgetic activity, most opioids have effects on many other physiologic processes, including respiration, temperature regulation, and gastrointestinal motility (Gilman et al. 1980). Some of the endogenous opioid peptides have also been implicated in mental illness (Verebey 1982).

The first opioid receptors were identified about 15 years ago (Lord et al. 1977; Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). They are now known to include several different types, including μ (selective for morphine), δ (enkephalins), and κ (ethylketocyclazocine). Still other receptor types, either in brain or in peripheral tissues (Grevel and Sadee 1983; Schulz et al.

¹The studies described in this paper were supported by NIDA research grant DA-00564. H.H.L. and N.M.L. are recipients of a research scientist award.

²Department of Pharmacology, University of California Medical Center, San Francisco, CA 94122.

1981), as well as subtypes for μ (Loew et al. 1986; Nishimura et al. 1984) and κ (Iyengar et al. 1986) receptors may exist.

Because of this heterogeneity, and because of the great difficulty in solubilizing opioid receptors in a form retaining binding activity, purification of these receptors has proceeded more slowly than that for other cell surface receptors. However, several groups have recently reported some success in solubilizing active receptors (Bidlack et al. 1981; Cho et al. 1986; DeMoliou-Mason and Barnard 1984; Gioannini et al. 1985; Maneckjee et al. 1985; Simonds et al. 1985). In some cases, certain receptor types have been distinguished (Chow and Zukin 1983; Crema et al. 1986; DeMoliou-Mason and Barnard 1986), and a few reports have claimed purification to near homogeneity (Cho et al. 1986; Gioannini et al. 1985; Simonds et al. 1985).

Using a combination of affinity chromatography, lectin chromatography, and gel filtration, we recently reported purification from bovine brain of a protein selective for opioid alkaloid ligands (Cho et al. 1986). A novel feature of this protein was that it required acidic lipids possessing unsaturated fatty acids in order to manifest binding activity; neither the protein nor the lipids alone exhibited significant opioid binding (Hasegawa et al. 1987). The binding affinities of ligands to this reconstituted material are lower than the corresponding values for binding to brain membranes, but the rank orders of binding affinities to the two preparations are highly correlated (Cho et al. 1986; Hasegawa et al. 1987).

Using standard procedures, very recently we succeeded in cloning the cDNA coding for this opioid-binding protein (Schofield et al. submitted). Here we present these results and discuss some of the structural interpretations they have enabled us to make.

Amino Acid Sequence of Opioid-Binding Protein

The amino acid sequence of a portion of the opioid-binding protein was determined, oligonucleotide probes corresponding to these sequences were synthesized, and these probes were used to screen a cDNA library from bovine brain. A single DNA sequence containing all sequences of the probes

M G V C G S L F F P Q P W K C L N V V V S R Q L R L L S A T L V P T G V P
 V R S G D R V A T F P K A M D N V T V A G N G E S A T L R C T I D
 D R V T R V A Q Y S L F P K N R S I M I V R I G D I S V P M A E F F N G A V V
 L V N T P T S R C L L E I T V P M A E F F N G A V V T A Y A V D P P T V K E Y E L S
 N H P K T S R C L L E I T V P M A E F F N G A V V T A Y A V D P P T V K E Y E L S
 S S V T L L E I T V P M A E F F N G A V V T A Y A V D P P T V K E Y E L S
 V S E D E K V K I S A S I T L Y G P I K F
 P D V R C E A S A I S T L Y G P I K F
 I L S E N K G H I S T L Y G P I K F
 R I E N K G H I S T L Y G P I K F
 K L G I T N A S I T L Y G P I K F
 L W L S G T L F A H F I K F

Figure 1.—Amino acid sequence of opioid-binding protein, designated by standard single-letter notation. Cysteine residues flanking the three Ig domains are boxed. Potential glycosylation sites, having the sequence N-X-S/T, are underlined.

was isolated. This sequence was determined and translated to give the amino acid sequence of the opioid-binding protein (figure 1).

The protein consists of 345 amino acids, for a calculated molecular size of 37.9 kilodaltons (kDa). The discrepancy between this value and the molecular size of the originally purified opioid-binding protein (58 kDa) suggests that the latter might be glycosylated. This conclusion is supported by two other observations: (1) the opioid-binding protein binds to lectin affinity columns (Cho et al. 1986), and (2) there are six potential glycosylation sites in the amino acid sequence of the protein, consisting of an asparagine residue in close proximity to a serine or threonine (figure 1, underlined sequences).

Opioid-binding Protein Is a Member of the Immunoglobulin Superfamily

A search of the NBRF-PIR data base revealed that our opioid-binding protein had significant homologies to several proteins (Schofield et al. submitted; table 1). The highest degree of homology was to two cell adhesion molecules, neural cell adhesion molecule (N-CAM) and myelin-associated glycoprotein (MAG); the sequences of both of these proteins are about 20 percent homologous with that of the opioid-binding protein (Schofield et al. submitted). Somewhat lower significance values, also suggestive of an evolutionary relationship, were found for the neural adhesion molecule (L1), as well as for receptors for interleukin-6 (IL-6) and platelet-derived growth factor (PDGF) and for the T-cell receptor alpha-chain precursor.

All of these proteins are members of the immunoglobulin (Ig) superfamily, a group of proteins characterized by repeating domains flanked by cysteine residues (Williams 1987). In a typical Ig domain, the cysteines are 50-60 amino acids apart and are thought to form internal disulfide bridges that are critical to the proper folding of the protein molecule. The domain usually begins with the sequence L-X-C, where X is any amino acid, and ends with the sequence Y-X-C (occasionally, L is replaced by a conservative substitution such as M, I, or V, and Y is substituted by F). Many members of

Table 1.—Homologies of opioid-binding protein with other proteins^a

Protein	Search score		z value	
	Initial	Aligned	Initial	Aligned
N-CAM	136	147	24.51	11.62
MAG	99	206	10.60	11.12
L1	75	117	6.67	6.83
IL-6 receptor	43	76	2.28	6.47
T-cell receptor	54	61	5.27	5.01
Inhibin	54	61	3.90	4.84
PDGF receptor	53	102	3.26	4.81
Kinase-related transforming	66	86	6.21	4.19
Link	62	69	6.32	4.10
IL-1 receptor	54	65	3.47	2.53
T-cell surface glycoprotein	59	60	4.98	2.42
I-CAM	38	69	0.57	2.48

^aThe amino acid sequence of the opioid-binding protein (figure 1) was compared with sequences in the NBRF-PIR data base using the FASTP program (Lipman and Pearson 1985). This algorithm does not compare the full sequences of the test protein and proteins in the data base but only a subsequence of highest similarity. The initial score is determined by totaling identical matches as well as conserved replacements, according to a standard amino acid weighting matrix (Dayhoff et al. 1983). The aligned score is determined by allowing for insertions and deletions in the two sequences being compared. Initial scores of greater than 50 and aligned scores of greater than 100 are usually considered suggestive of an evolutionary relationship (Lipman and Pearson 1985). The proteins listed were subsequently compared by using the RDF test for sequence similarity (Lipman and Pearson 1985). In this test, the alignment score of each pair is compared with the mean of a similarly derived set of scores derived by comparing 20 randomized versions of each sequence. Significance scores (z values) are defined as the number of standard deviations by which the aligned score of the original pair exceeds the mean of the randomized aligned scores. A z value of 6 or greater is suggestive of an evolutionary relationship between two proteins of undetermined functional similarity (Lipman and Pearson 1985).

the Ig superfamily have multiple Ig domains, and three such domains are present in our opioid-binding protein (figure 1; the flanking cysteine residues are boxed).

Detailed analysis of the Ig superfamily suggests that Ig domains are of three types, known as the V set, C1 set, and C2 set (Williams 1987). The V domains are longer than the C domains and are thought to contain an extra loop, which forms a second variable region in antibodies.

To determine the most appropriate classification of our opioid-binding protein, each of the domains was compared with a set of typical V, C1 and C2 sequences, using the RDF program associated with the FASTP alignment algorithm of Lipman and Pearson (1985). This program uses an amino acid weighting matrix that gives positive scores not only for identical amino acids in a pair of sequences being compared but also for conserved amino acid replacements (Dayhoff et al. 1983). The alignment score for each pair of sequences is compared with the mean of scores obtained by aligning 20 randomizations of each sequence, with the significance value (z value) of a given score defined as the number of standard deviations above the mean of randomized scores. Since only members of a single superfamily are being compared, z values of greater than 3 are considered highly suggestive of an evolutionary relationship.

Comparison of each of these domains with typical V, C1, and C2 domains revealed that each of these domains was most closely related to the C2 set (table 2). Thus, domains I, II, and III had z scores of 3 or more (table 2, boxed values) when compared with 9, 12, and 11, respectively, of the 13 members chosen to represent this set. In addition, however, domain III showed a strong relationship with the V set (five of seven scores greater than 3), and domain II showed a significant relationship with the C1 set (four of nine scores greater than 3). Finally, each of the three domains of the opioid-binding protein showed significant homology with each of the others (z values were 4.37 [I versus II]; 4.79 [I versus III]; and 2.85 [II versus III]). As a comparison, only about 2 percent of randomly selected sequence pairs yield a score of 3 or better on this test (William 1987).

Table 2.—Homologies of opioid-binding protein domains with typical V, C1, and C2 domains^a

Set	Domain		
	I	II	III
V			
Ig V lambda	1.50	1.89	3.09
Ig V heavy	1.26	-0.75	1.27
MRC OX-2 (I)	1.36	0.04	2.31
Poly IgR (III)	12.49	2.17	4.01
Tcr V alpha	-0.11	2.17	3.33
Tcr V beta	2.81	2.93	4.69
Thy-1	2.50	3.47	3.12
C1			
Beta-2m	0.44	0.30	0.68
IG C heavy (I)	1.83	4.05	-1.01
IG C heavy (III)	1.10	3.20	-1.18
Ig C kappa	-1.73	-0.84	2.55
Ig C lambda	1.96	3.03	1.36
MHC I alpha 3	4.48	2.87	1.20
MHC II beta 2	2.17	0.64	7.80
Tcr C beta	0.31	4.82	1.26
Tcr C gamma	0.92	1.73	0.42

^aImmunoglobulin domains were defined as extending for 20 amino acids on either side of flanking cysteine residues (Williams 1987). The significance of homologies between domains was determined by the RDF program associated with the FASTP alignment algorithm (Lipman and Pearson 1985), using the scoring matrix of Dayhoff et al. (1983). Significance scores (z values) are defined as the number of standard deviations by which the alignment score of any two domains exceeds the mean of alignment scores calculated from 20 randomized versions of these sequences. Abbreviations used are as in Williams (1987), and sources of sequences are the NBRF data bank except for L1 (neural adhesion molecule; Moos et al. 1988), IL-1 R (interleukin-1 receptor; Sims et al. 1988), and IL-6 R (interleukin-6 receptor; Yamasaki et al. 1988).

Table 2.—Homologies of opioid-binding protein domains with typical V, C1, and C2 domains—Continued

Set	Domain		
	I	II	III
C2			
N-CAM (IV)	0.63	9.85	32.88
MAG (III)	4.63	21.21	8.44
MAG (IV)	3.97	13.35	3.06
PDGF R (III)	7.25	10.18	7.64
Alpha-1 B-GP (III)	5.17	3.43	3.13
L1 (I)	0.74	3.90	13.30
L1 (II)	5.79	6.06	0.75
L1 (III)	1.84	12.49	17.44
L1 (IV)	3.82	7.36	1.20
L1 (V)	5.73	13.65	11.98
L1 (VI)	6.67	8.57	6.07
IL-1 R (II)	2.90	5.13	6.57
IL-6 R (I)	5.02	1.35	7.91

Thus, the sequence of this opioid-binding protein is generally consistent with the notion that it is a functionally relevant receptor, although it conceivably may also play a role in cell adhesion. Nevertheless, homology of this opioid-binding protein with the Ig superfamily is somewhat surprising, because some opioid receptors, namely the δ type in NG108-15 neuroblastoma x glioma hybrid cells and in mammalian striate, are coupled via a G protein to adenylate cyclase (Sharma et al. 1975; Koski et al. 1982). This finding has suggested that these receptors are structurally and functionally related to a group of cell surface receptors that are associated with G proteins, which include the β_1 -, β_2 -, and α_2 -adrenergic, muscarinic cholinergic receptors and serotonin 1c receptors (Dixon et al. 1986; Julius et al. 1988; Kobilka et al. 1987; Kubo et al. 1986; Peralta et al. 1987). All of these receptors have several hydrophobic regions in their interior, which are thought to span the membrane.

Although the second messenger(s) mediating the actions of our opioid-binding protein has not yet been determined, its sequence suggests either that it does not interact with G proteins, or if it does, that this interaction is quite distinct from that of receptors with multiple membrane-spanning regions. An alternative function is suggested by the observation that several members of the Ig superfamily with which our opioid-binding protein shares significant homology, such as the PDGF receptor, possess tyrosine kinase activity. Activation of these receptors results in phosphorylation of several intracellular substrates and of the receptor itself, and in some cases may also induce a number of other cellular responses. However, our opioid-binding protein does not possess a consensus sequence that is thought to contain the tyrosine kinase activity.

Functional Relevance of Opioid-Binding Protein

To establish that a cDNA sequence actually codes for a functional protein, it is conventional to transfect that cDNA into a cell line not containing that protein and demonstrate the presence of the appropriate functional activity. To date, we have not been able to demonstrate opioid-binding activity in cells transfected with cDNA of our opioid-binding protein. This may be because the transfected cDNA is not expressed.

As an alternative approach, however, we have used antibodies to demonstrate the functional relevance of our cDNA. First, we prepared an antibody against a peptide corresponding to a portion of the predicted amino acid sequence of the cDNA. This antibody was used to construct an immunoaffinity column, which specifically absorbed a portion of material from Triton-solubilized brain membranes. This material bound opioids; furthermore, opioid-binding to this material was inhibited by a monoclonal antibody that was originally prepared against the purified opioid-binding protein (Roy et al. 1988).

These results indicate that the protein coded by our cDNA plays an important role in opioid binding. Interestingly, although the original purified protein has selectivity for alkaloid ligands (Cho et al. 1986), monoclonal antibodies to this protein are able to inhibit binding of μ , δ , and κ opioids to brain membranes (Roy et al. 1988). This result, together with the

observation that the monoclonal antibody inhibits binding in a noncompetitive fashion, suggests that this protein may be a single component of a larger, multisubunit complex. In this view, while the isolated protein would contain the opioid-binding site, the *selectivity* of this site would be determined, at least in part, by other subunits with which it was associated. This interpretation, which suggests that different opioid receptor types such as μ , δ , and κ contain a common binding site sequence, is also consistent with the observation that as much as 80-90 percent of the opioid binding of brain membranes is absorbed by the immunoaffinity column constructed with antibodies against a portion of the predicted protein sequence of the cDNA (Roy et al. unpublished data).

Of those surface membrane receptors that have been well characterized, the nicotinic cholinergic receptor provides the best model of a system containing interacting binding and nonbinding subunits (Changeux and Revah 1987). This receptor contains five subunits, two α , one β , one γ , and one δ ; the α subunits contain binding sites for nicotinic cholinergic ligands, and recently it has been shown that the γ subunits also contain binding sites. The five subunits together are inserted into the cell membrane so as to form an ion channel, and the nonbinding subunits are thought to regulate the function of cholinergic ligands allosterically.

Although the amino acid sequence of our opioid-binding protein clearly places it in a different class of proteins from the nicotinic receptor (see above), it is noteworthy that several studies have demonstrated that opioid receptors in some systems are coupled to ion channels and that different receptor types are coupled to different channels (Cherubini and North 1985). However, proof of this hypothesis will require isolation and sequencing of these putative other subunits.

Is Opioid-binding Protein Linked to the Cell Membrane by Glycosyl-Phosphatidylinositol?

Conventionally, membrane-bound proteins have been thought to consist of two general classes, peripheral and integral (Singer and Nicolson 1972). Peripheral proteins lie largely or entirely on the surface of the membrane and are bound by electrostatic bonds; integral proteins have significant portions

buried in the lipid bilayer, frequently spanning it, where they interact by hydrophobic forces.

Most cell surface receptors characterized to date are thought to be integral proteins and contain multiple regions that span the membrane. The best established of these belong to the rhodopsin superfamily, receptors that are coupled to GTP-binding proteins in the membrane; as noted earlier, they include the β_1 -, β_2 -, and α_2 -adrenergic, muscarinic cholinergic receptors, substance K, and serotonin 1c receptors. Another group of transmembrane receptors, including receptors for nicotinic cholinergic ligands and for γ -aminobutyric acid (GABA) (Schofield et al. 1987), make up the so-called ligand-gated receptor superfamily.

Recently, however, a new class of membrane proteins has been described, members of which are linked to the membrane by glycosylphosphatidylinositol (PI) (Low and Saltiel 1988). This group includes hydrolytic enzymes such as alkaline phosphatase, acetylcholinesterase, and 5'-nucleotidase, several cell adhesion molecules such as N-CAM, and several other types of proteins, many of unknown function. All of these proteins can be released from the membrane by treatment with PI-specific phospholipase C (PLC) or phospholipase D (PLD), yielding the PI-glycan linked by ethanolamine to the C terminus.

Although no major structural or functional properties have been found that are shared by all members of this group, all lack the internal hydrophobic sequences of amino acids found in integral membrane receptors, instead possessing a short hydrophobic sequence at the C terminus. Our opioid-binding protein also has these features; together with the fact that several PI-linked proteins are also members of the Ig superfamily, including N-CAM and the Thy-1 and Qa antigens, this finding suggests that our opioid-binding protein may also be linked to the membrane in this fashion. We are currently carrying out experiments to test this hypothesis.

If our opioid-binding protein is linked to the membrane by PI-glycan, it would be the first receptor known to be attached in this fashion. This mode of attachment may have several important implications for its function. Most important, the products of protein cleavage from the membrane, either diacylglycerol (DAG; produced by PLC cleavage) or phosphatidic acid

(produced by PLD cleavage), are thought to act as second messengers in the actions of certain receptors, particularly DAG, which activates protein kinase C. Thus, receptor release could be directly linked to the activation of other cellular processes. In addition, the PI-glycan linkage may greatly increase the mobility of the receptor, which may in turn promote coupling with second messengers.

Summary

Although opioid receptors were identified 15 years ago, only very recently have laboratories begun to report their purification and molecular characterization. We have purified to homogeneity a 58-kDa protein that binds opioids with high affinity and with rank order correlating with that of membrane-bound opioid receptors, although this binding requires the presence of acidic lipids. We believe that our cloning of this protein represents the first successful cloning of a gene for a putative opioid receptor.

The predicted sequence of this protein suggests that it is a glycoprotein that is largely extracellular in orientation. In addition, it may be linked to the cell membrane by a phosphatidyl-glycosyl group, which suggests many possibilities for both its ligand-mediated functions and its regulation. This protein has significant homologies with certain members of the immunoglobulin superfamily, such as N-CAM, MAG, and the PDGF and IL-1 receptors, which suggests that it could have multiple functions, both as a receptor and as a cell contact molecule.

Future work will focus on the second messengers associated with this opioid-binding protein and will also attempt to determine its relationship to the convention μ , δ , and κ opioid receptors. The possibility that this protein represents a common binding subunit shared by different opioid receptor types is a particularly important hypothesis raised by our work and one that should be testable in the near future.

References

- Bidlack, J.M.; L.G. Abood; P. Osei-Gyimah; and S. Archer. Purification of the opiate receptor from rat brain. *Proc Nat Acad Sci USA* 78:636-639, 1981.
- Changeux, J.-P., and Revah, F. The acetylcholine receptor molecule: Allosteric sites and the ion channel. *Trends Neurosci* 10:245-25, 1987.
- Cherubini, E., and North, R.A. Mu and kappa opioids inhibit neurotransmitter release by different mechanisms. *Proc Nat Acad Sci USA* 82:1860-1863, 1985.
- Cho, T.M.; J. Hasegawa; B.L. Ge; and H.H. Loh. Purification to apparent homogeneity of a mu-specific opioid receptor from rat brain. *Proc Nat Acad Sci USA* 83:4138-4142, 1986.
- Chow, T., and R.S. Zukin. Solubilization and preliminary characterization of mu and kappa opiate receptor subtypes from rat brain. *Mol Pharmacol* 24:203-212, 1983.
- Crema, G.; T.L. Gioannini; J.M. Hiller; and E.J. Simon. The direct demonstration of binding of mu, delta, and kappa agonists to digitonin-solubilized opioid receptors from bovine striatum. *INRC Abstr* P-7, 1986.
- Dayhoff, M.O.; W.C. Barker; and L.T. Hunt. Establishing homologies in protein sequences. *Methods Enzymol* 91:524-545, 1983.
- DeMoliou-Mason, C.D., and E.A. Barnard. Solubilization in high yield of opioid receptors retaining high affinity delta, mu and kappa binding sites. *FEBS Lett* 170:378-382, 1984.
- DeMoliou-Mason, C.D., and E.A. Barnard. Characterization of opioid receptor subtypes in solution. *J Neurochem* 46:1129-1136, 1986.
- Dixon, R.A.F.; B.K. Kobilka; D.J. Strader; J.L. Benovic; H.G. Dohlman; T. Frielle; M.A. Bolanowski; C.D. Bennett; E. Rands; R.E. Diehl; R.A. Mumford; E.E. Slater; I.S. Sigal; M.G. Caron; R.J. Lefkowitz; and C.D.

- Strader. Cloning of the gene and cDNA for mammalian β -adrenergic receptor and homology with rhodopsin. *Nature* (London) 321:75-79, 1986.
- Gilman, A.G.; L.S. Goodman; and A. Gilman. *The Pharmacological Basis of Therapeutics*. New York: Macmillan, 1980.
- Gioannini, T.L.; A.D. Howard; J.M. Hiller; and E.J. Simon. Purification of an active opioid-binding protein from bovine striatum. *J Biol Chem* 260:15117-15121, 1985.
- Grevel, J.T., and W. Sadee. An opiate binding site in the rat brain is highly selective for 4,5-epoxymorphinans. *Science* 221:1198-1201, 1983.
- Hasegawa, J.; H.H. Loh; and N.M. Lee. Lipid requirement for mu opioid receptor binding. *J Neurochem* 49:1007-1012, 1987.
- Iyengar, S.; H.S. Kim; and P.L. Wood. Effects of kappa opiate agonists on neurochemical and neuroendocrine indices: Evidence for kappa receptor subtypes. *J Pharmacol Exp Ther* 238:429-436, 1986.
- Julius, D.; A.B. MacDermott; R. Axel; and T.M. Jessell. Molecular characterization of a functional cDNA encoding the serotonin 1c receptor. *Science* 241:558-564, 1988.
- Kobilka, B.K.; H. Matsui; T.S. Kobilka; T.L. Yang-Feng; U. Francke; M.G. Caron; R.J. Lefkowitz; and J.W. Regan. Cloning, sequencing, and expression of the gene coding for the human platelet α -adrenergic receptor. *Science* 238:650-656, 1987.
- Koski, G.; Streaty, R.A.; and Klee, W.A. Modulation of sodium-sensitive GTPase by partial opiate agonists: An explanation for the dual requirement for Na^+ and GTP in inhibitory regulation of GTPase. *J Biol Chem* 257:14035-14040, 1982.
- Kubo, T.; K. Fukuda; A. Mikami; A. Maeda; H. Takahashi; M. Mishina; T. Haga; K. Haga; A. Ichiyama; K. Kangawa; M. Kojima; H. Matsuo; T. Hirose; and S. Numa. Cloning, sequencing and expression of complementary DNA encoding the muscarinic acetylcholine receptor. *Nature* (London) 323:411-416, 1986.

- Lipman, D.J., and W.R. Pearson. Rapid and sensitive protein similarity searches. *Science* 227:1435-1441, 1985.
- Loew, G.; C. Keys; B. Luke; W. Polgar; and L. Toll. Structure-activity relationships of morphiceptin analogs: Receptor binding and molecular determinants of mu-affinity and selectivity. *Mol Pharmacol* 29:546-553, 1986.
- Lord, J.A.H.; A.A. Waterfield; J. Hughes; and H.W. Kosterlitz. Endogenous opioid peptides: Multiple agonists and receptors. *Nature* (London) 267:495-499, 1977.
- Low, M.G., and A.R. Saltiel. Structural and functional roles of glycosyl-phosphatidylinositol in membranes. *Science* 239:268-275, 1988.
- Maneckjee, R.; R.S. Zukin; S. Archer; J. Michael; and P. Osei-Gyimah. Purification and characterization of the mu opioid receptor from rat brain using affinity chromatography. *Proc Natl Acad Sci USA* 82:594-598, 1985.
- Moos, H.; Tacke, R.; Scherer, H.; Teplow, D.; Fruh, K.; and Schachner, M. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. *Nature* (London) 334:701-703, 1988.
- Nishimura, S.C.; L.D. Recht; and G.W. Pasternak. Biochemical characterization of high-affinity ^3H -opioid binding. Further evidence for μ_1 sites. *Mol Pharmacol* 25:29-37, 1984.
- Peralta, E.G.; J.W. Winslow; G.L. Peterson; D.H. Smith; A. Ashkenazi; J. Ramachandran; M.I. Schimerlik; and D.J. Capon. Primary structure and biochemical properties of an m_2 muscarinic receptor. *Science* 236:600-605, 1987.
- Pert, C.B., and S.H. Snyder. Opiate receptor: Its demonstration in nervous tissue. *Science* 179:1011-1014, 1973.

- Roy, S.; Zhu, Y.X.; Loh, H.H.; and Lee, N.M. A monoclonal antibody that inhibits opioid binding to rat brain membranes. *Biochem Biophys Res Commun* 154:688-693, 1988.
- Schofield, P.R.; M.G. Darlison; N. Fujita; D.R. Burt; F.A. Stephenson; H. Rodriguez; L.M. Rhee; J. Ramachandran; V. Reale; T.A. Glencorse; P.H. Seeburg; and E.A. Barnard. Sequence and functional expression of the GABA_A receptor shows a ligand-gated receptor super-family. *Nature* (London) 328:221-227, 1987.
- Schulz, R.; M. Wuster; and A. Herz. Pharmacological characterization of the epsilon-opiate receptor. *J Pharmacol Exp Ther* 216:604-606, 1981.
- Sharma, S.; Nirenberg, M.; and Klee, W. Morphine receptors are regulators of adenylate cyclase activity. *Proc Natl Acad Sci USA* 72:590-594, 1975.
- Simon, E.J.; J.M. Hiller; and J. Edelman. Stereospecific binding of the potent narcotic analgesic [3H] etorphine to rat brain homogenate. *Proc Natl Acad Sci USA* 70:1947-1949, 1973.
- Simonds, W.F.; T.R. Burke; K.C. Rice; A.E. Jacobson; and W.A. Klee. Purification of the opioid receptor of NG108-15 neuroblastoma x glioma hybrid cells. *Proc Natl Acad Sci USA* 82:4774-4978, 1985.
- Sims, J.E.; March, C.J.; Cosman, D.; Widmer, M.B.; MacDonald, H.R.; McMahan, C.J.; Grubin, C.E.; Wignall, J.M.; Jackson, J.L.; Call, S.M.; Friend, D.; Alpert, A.R.; Gillis, S.; Urdal, D.L.; and Dower, S.K. cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. *Science* 241:585-589, 1988.
- Singer, S.J., and G.L. Nicolson. The fluid mosaic model of membrane structure. *Science* 175:720-731, 1972.
- Terenius, L. Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta Pharmacol (Kbh)* 32:317-320, 1973.

Verebey, K., ed. *Opioids in Mental Illness: Theories, Clinical Observations and Treatment Possibilities*. Vol. 398. *Ann NY Acad Sci*, 1982.

Williams, A.F. A year in the life of the immunoglobulin superfamily. *Immunol Today* 8:298-303, 1987.

Yamasaki, K.; Taga, T.; Hirata, Y.; Yawata, H.; Kawanishi, Y.; Seed, B.; Taniguchi, T.; Hirano, T.; and Kishimoto, T. Cloning and expression of the human interleukin-6 (BSF-2/IFN β 2) receptor. *Science* 241:825-828, 1988.

Discussion

HOFFMAN: When you screened the expression library for the mu receptor, did you see anything that looked like the delta receptor?

LOH: No, the mu receptor was isolated completely differently. We isolated the binding protein and cloned the gene.

HOFFMAN: But you had a probe for the mu receptor, and I wondered if you might have picked up other clones. That is, if there is homology between the mu and delta receptors.

LOH: No, we did not do it that way.

TABAKOFF: Did you try to express the mu receptor?

LOH: Yes, we have tried very hard and while we can express the receptor and it is in the membrane, we have been unable to show binding. We know it is there by Western blot. However, you are asking the oocyte (or other cell) to do many things. For example, one third of the receptor is sugar, and we are asking the oocyte (or other cells) not only to express the protein but to also glycosylate it and insert it into the proper lipid environment.

TABAKOFF: That is interesting because in the isolation of the clone you used a probe that was based on the sequence in the binding region. The sugar, as you say, must be very important to the binding.

LOH: Yes, indeed.

DEITRICH: I didn't understand why you had to use a eukaryotic cell to express the delta receptor. Can't you use another cell type?

LOH: It would be very nice to express it in *E. coli*, but we have not attempted that. It is convenient to use the C6 glioma cell because we know that it has the catalytic subunit of adenylate cyclase and the G protein. The opiate receptor is coupled to that.

Corticotropin-Releasing Factor, Stress, and Ethanol¹

George F. Koob², Karen T. Britton³, and Cindy L. Ehlers²

Introduction

Corticotropin-releasing factor (CRF), a 41-amino-acid peptide (Vale et al. 1981), has potent activating effects on the pituitary-adrenal axis, as shown by its ability to release adrenocorticotropin hormone (ACTH) and β -endorphin from the anterior pituitary. CRF is presumably the long-sought hypothalamic neurohumor with the specific function of releasing ACTH from the anterior pituitary and thus may be considered the final common pathway for the neurohumoral control of ACTH.

The significance of the chemical identification of CRF can perhaps be best understood in terms of classical "stress" theory. Initially formulated by Selye in 1936, the general concepts of stress theory have undergone some refinement over the past 40 years. A recent definition of stress that includes the role of antecedent psychologic stimuli is that "stress is anything which causes an alteration of psychological homeostatic processes" (Burchfield

¹Preparation of this chapter was supported in part by NIH grants AM 26741, AA 00098, and AA 06059 (to C.L.E.). This is publication no. 5622 BCR from the Research Institute of Scripps Clinic, La Jolla, CA.

²Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

³Department of Psychiatry, San Diego Veterans Administration Medical Center and UCSD School of Medicine, La Jolla, CA 92161.

1979). Probably the most reliable indication that a state of stress exists is variation in the production of ACTH. Internal or external demands are conveyed in the form of stimuli to the anterior pituitary via neurohumoral means (presumably CRF), and the pituitary responds with a secretion of ACTH. ACTH, in turn, stimulates the adrenal cortex to secrete glucocorticoids, which have widespread effects on metabolism, such as gluconeogenesis, hyperinsulinemia, lysis of lymphoid tissue, increased gastric secretion, and reduced inflammatory and antibody responses. These physiologic changes in response to increased hypothalamic-pituitary action are paralleled by alterations in behavior that have been associated with increases in alertness and attention (De Wied 1977).

Thus, an alternative means by which behavioral or physiologic responses to stress or anxiety might be mediated by the hypothalamic-pituitary system in an organism is via direct neurotropic action of CRF in the central nervous system (CNS) itself. Thus, just as pathways project to the hypothalamus from the limbic areas to activate via CRF the pituitary-adrenal axis, so might CRF feed back to these same areas to mediate appropriate behavioral responses to stress.

Behavioral Activation Produced by CRF

CRF has central neurotropic properties when administered directly into the CNS. Intraventricular injection of CRF produces elevation of plasma epinephrine, norepinephrine, and glucose (Brown et al. 1982a-c). These effects are reproducible in hypophysectomized animals but are abolished by ganglionic blocking agents (Brown and Fisher 1983). CRF injected intracereboventricularly (ICV) also produces a profound dose-dependent activation of the electroencephalogram (EEG) (Ehlers et al. 1983). Doses of 0.015-0.15 nmol produce a long-lasting activation of EEG, and after a 2-hr delay, some interictal spikes occur in the amygdala and hippocampus. Higher doses (1.5-3.75 nmol) are characterized by consistent amygdala interictal spikes and afterdischarges and, after a delay of 4-7 hr, some motor seizures. These seizures develop over time in a manner not unlike those produced by amygdala "kindling" paradigms. At the cellular

level, CRF produces increases in the firing frequencies of cells within the locus coeruleus (Valentino et al. 1983), a system thought to be of importance in the mechanisms by which the brain is able to attend selectively to certain novel external events.

The autonomic and electrophysiologic activation produced by central administration of CRF is paralleled by a dose-dependent locomotor activation (Sutton et al. 1982; Koob et al. 1984a). These effects appear to be independent of direct mediation by the pituitary-adrenal system, since they were observed in hypophysectomized and dexamethasone-treated rats (figure 1). Taken together with the fact that this activation is seen only with central administration, these observations suggested that CRF exerts its effects within the CNS.

Although other peptides such as the endorphins and ACTH have been shown to produce increases in spontaneous behavioral activity, the nature of the CRF response differs substantially from the response observed with these other peptides. For example, CRF does not produce the initial depressant phase followed by bursts of locomotor activity that characterizes ICV injections of opioid peptides (Segal et al. 1979); also, the locomotor activation caused by CRF is not antagonized by the opiate antagonist naloxone or by low doses of the dopamine receptor antagonist alpha flupenthixol (Koob et al. 1984a), nor is it reversed by 6-hydroxydopamine lesions of the region of the nucleus accumbens, lesions that reverse the locomotor-stimulated effects of indirect sympathomimetics (Swerdlow and Koob 1985). Injected centrally, ACTH itself fails to increase locomotor activity but instead produces an increase in grooming behavior, as has been observed by others (Gispen et al. 1975).

CRF Increases "Emotionality"

Perhaps of more importance for the conceptualization of CRF as a peptide involved in the organism's behavior response to stress are the experiments showing that CRF can potentiate the effects of exposure to a novel, presumably aversive environment. Rats tested in a novel open field after ICV injection of doses of CRF (0.0015-0.15 nmol) showed responses consistent with an increased "emotionality" or increased sensitivity to the

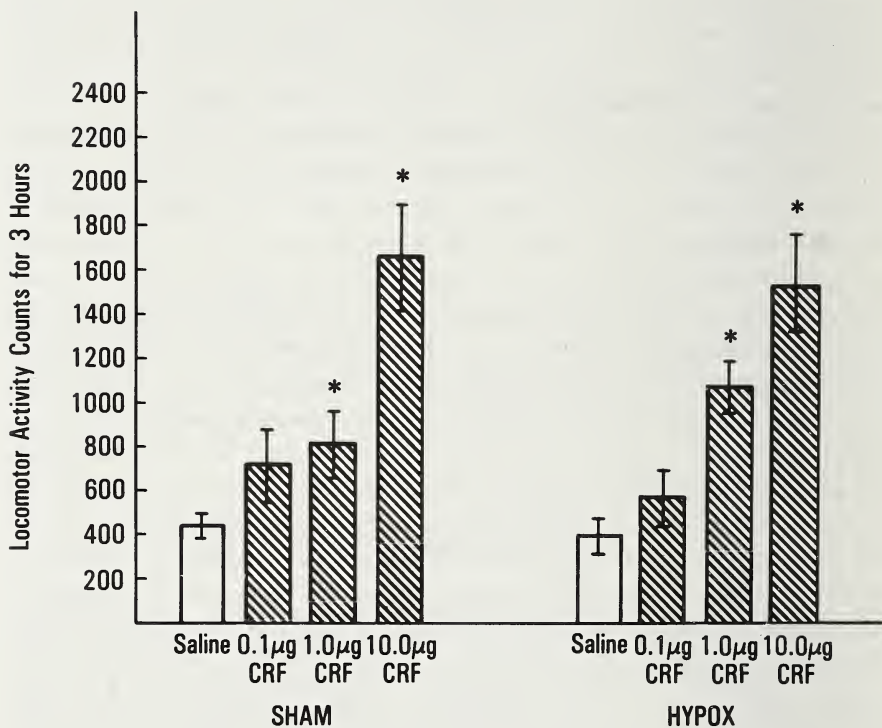


Figure 1.—Effects of CRF injected ICV on locomotor activity in photocell cages in hypophysectomized rats treated chronically with rat growth hormone and in sham-operated animals. Results represent the total activity counts over 3 hr (mean \pm standard error; $n=9$ in each group). *, Significantly different from saline injection. $P < 0.05$ main effects analysis of variance (ANOVA). Reprinted with permission from *Peptides*, 6:923-926, 1985. Eaves, M.; Britton, K.T.; Rivier, J.; Vale, W.; and Koob, G.F. Effects of corticotropin releasing factor on locomotor activity in hypophysectomized rats. Copyright 1985, Pergamon Press plc.

stressful aspects of the situation, as rats showed *decreases* in locomotion and rearing. In this open-field test, a typical saline-injected rat rapidly circled the outer squares of the open field during the first 3-4 min of the 5-min test. During the last 1-2 min of the test, these saline-injected animals then made some forays into the center of the open field, usually accompanied by rearing on their hindlegs. Typically, a rat injected with 0.15 nmol of CRF and placed 60 min later in the open field moved hesitantly to the outer squares and then either circled the open field, remaining close to the floor, or remained in one of the corners, grooming or hesitantly moving forward and backward (Sutton et al. 1982)

In an operant conflict test, CRF produced a significant decrease in punished responding, a main effect opposite that observed with benzodiazepines. In addition, this "anxiogenic" effect was reversed by concomitant treatment with a benzodiazepine (Britton et al. 1985) (figure 2). However, this increased sensitivity to aversive events was not paralleled by an increased sensitivity to pain. Similar interactions with benzodiazepines were observed with CRF in the acoustic startle response (Swerdlow et al. 1986). CRF (1 μ g ICV) potentiated the acoustic startle response, and this effect also was doses-dependently reversed by chlordiazepoxide (Swerdlow et al. 1986).

Behavioral Effects of CRF Antagonist

Although the effects of exogenous CRF in potentiating behavior responses to stress are interesting, the hypothesis that behavior responses to stress depend on activation of endogenous CRF in the brain would be bolstered by evidence of an antistress effect of an antagonist to CRF. A peptide antagonist to CRF, α -helical CRF 9-41 (Rivier et al. 1984), has recently been shown to have behavioral actions in some test. This antagonist is 10 times less potent than CRF in binding to CNS CRF receptors (De Souza 1987). α -Helical CRF can reverse the effects of CRF (figure 3), and it has been shown to partially reverse stress-induced decreases in feeding (Krahn et al. 1986). This antagonist also appears to have activity in various open-field tests. α -Helical CRF injected into mice reverses stress-induced changes in a novel environment (multicompartment chamber) (Berridge and Dunn 1987) and

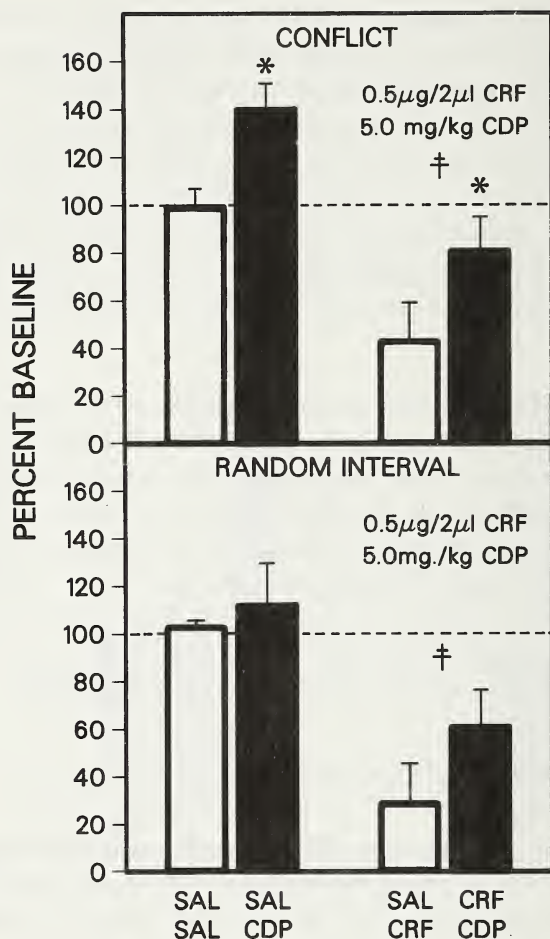


Figure 2.—Interaction of 0.5 μ g of CRF injected ICV and of 5.0 mg/kg of chlordiazepoxide (CDP) injected IP on responsiveness during the random interval and conflict components of an operant conflict test. Results are expressed as percent of baseline from the previous day. *, Significantly different from saline, main effect CDP; †, Significantly different from saline, main effect CRF, $P < 0.05$, two-way ANOVA. Reprinted with permission from *Psychopharmacology* 86:170-174, 1985. Britton, K.; Morgan, J.; Rivier, J.; Vale, W.; and Koob, G.F. Chlordiazepoxide attenuates CRF-induced response suppression in the conflict test. Copyright 1985, Springer-Verlag, Heidelberg.

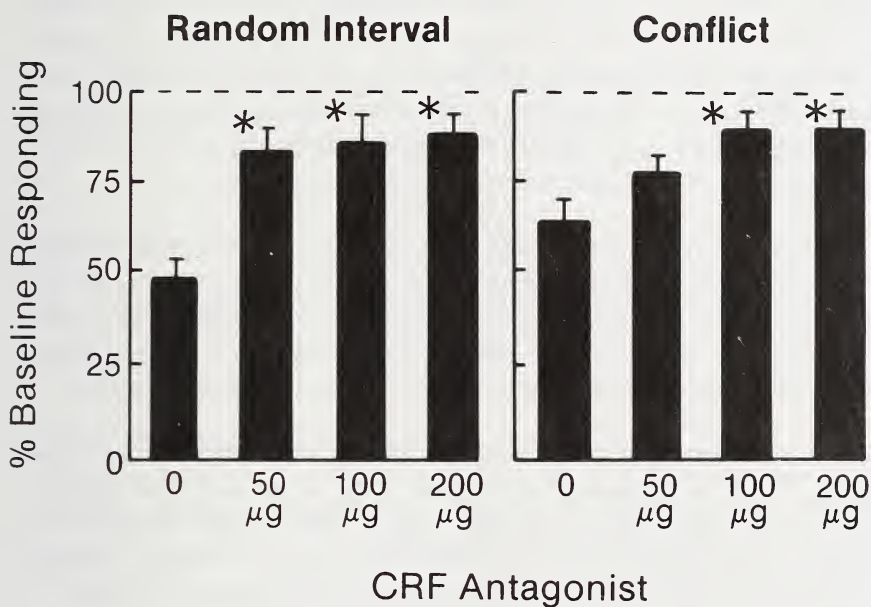


Figure 3.—Effect of CRF receptor antagonist (50, 100, or 200 µg ICV) on CRF-induced (1.0 µg ICV) response suppression in the conflict test. Values are expressed as percent baseline responding from the previous 2 days. *, Significantly different from controls, $P < 0.05$ (Neuman-Keuls); $n=12$ for saline; $n=10$, 11, and 11 for the three doses of CRF antagonist. Reprinted with permission from *Brain Research* 369:303-306, 1986. Britton, K.; Lee, G.; Vale, W.; Rivier, J.; and Koob, G.F. Corticotropin releasing factor antagonists block activating and "anxiogenic" actions of CRF in the rat. Copyright 1986, Elsevier Science Publishers BV, Amsterdam, The Netherlands.

also decreases the latency for rats to emerge into a novel open field from a dark, familiar enclosure (Takahashi et al. 1988).

However, these results with the CRF antagonist in aversive situations are reasonably subtle, particularly in light of a significant amount of negative results in some classic animal models sensitive to antianxiety drugs. α -Helical CRF given ICV alone does not produce a reliable release of punished responding (Britton et al. 1986), nor does it block the effects of shock in a conditioned emotional response (Cole et al. unpublished data).

Rats trained in avoidance tasks that received 0.1 and 1.0 μ g of CRF showed extreme "emotionality" for long periods after testing. When returned to their home cages, these rats also assumed boxing positions, vocalized, and fought with other rats for hours after testing. This unusual behavior was never observed in rats receiving these doses of CRF without avoidance experience.

This unusual interaction of CRF with the stress produced by aversive events suggested that a systematic investigation using a stress-induced fighting paradigm might exaggerate this phenomenon. This fighting test has been previously shown to be sensitive to environmental parameters such as stress level (Tazi et al. 1985) and to be modulated by peptides (Tazi et al. 1983).

Adult Wistar male rats (180-200 g), previously implanted with ICV cannula, were exposed in weight-matched pairs to mild inescapable electric foot shock during daily consecutive sessions. In such a paradigm, upright postures (boxing positions) or fighting (upright postures followed by physical contacts) develop within a few sessions. These behaviors depended on many environmental parameters, including the size of the test chamber and mainly the shock intensity (Tazi et al. 1983). Thus, the frequency of fighting responses is increased gradually with the shock intensity, probably as a consequence of the gradual increase in physiologic changes accompanying the increased stress.

Rat CRF at doses of 0, 0.01, 0.1, and 1.0 μ g was injected ICV in each of the two rats of a pair 30 min before every session. CRF increased the frequency of these responses in a dose- and shock-dependent manner. At the lower shock intensities (0.3 and 0.4 mA), there was no fighting between the control

rats. However, "boxing positions" were observed at these shock levels in control rats, and "boxing" was increased significantly by 0.1 and 1.0 μg of CRF. CRF doses of 0.01 and 0.1 μg significantly facilitated fighting at the 0.5-mA shock level. CRF at the highest dose and highest shock level totally disrupted the behavior of the animals.

More important, however, a slightly higher shock level (0.6 mA) produced a higher fighting frequency in control animals, and this fighting was reversed by 5 and 25 μg of α -helical CRF 9-41, a CRF antagonist (Tazi et al. 1987). These rats had not received exogenously administered CRF. This CRF antagonist has been shown to decrease both in vitro and in vivo baseline release of ACTH as well as ether-induced ACTH secretion (Rivier et al. 1984). These results suggest that under certain conditions of high arousal and stress, endogenous CRF systems may play a role in mediating behavioral responses.

CRF-Ethanol Interactions

Low doses of alcohol (0.5-1.0 g/kg intraperitoneally [IP]) produce a dose-dependent release of punished responding, as reflected by an increase in lever pressing in an incremental shock conflict test (Aston-Jones et al. 1984; Koob et al. 1984b). The same doses of alcohol produce a dose-dependent decrease in responding during the unpunished component, which presumably reflects the acute motor-debilitating effects of alcohol (Aston-Jones et al. 1984; Koob et al. 1984b). It was therefore of some interest to determine what interaction CRF would have with ethanol, given that CRF has proconflict effects by itself.

Alcohol in a dose of 0.75 g/kg reversed the suppressive effects of 0.5 μg of CRF injected ICV on punished responding but augmented the suppression of unpunished responding by CRF (Britton and Koob 1986) (figure 4). Results suggest that one mechanism for the tension-reducing properties of acute alcohol intoxication may involve a suppression of brain CRF systems.

Chronic ethanol ingestion has been demonstrated to produce an activating effect on the hypothalamic-pituitary-adrenal (HPA) axis in both clinical and animal studies (Van Thiel 1983). In animal studies, dose-related increases in

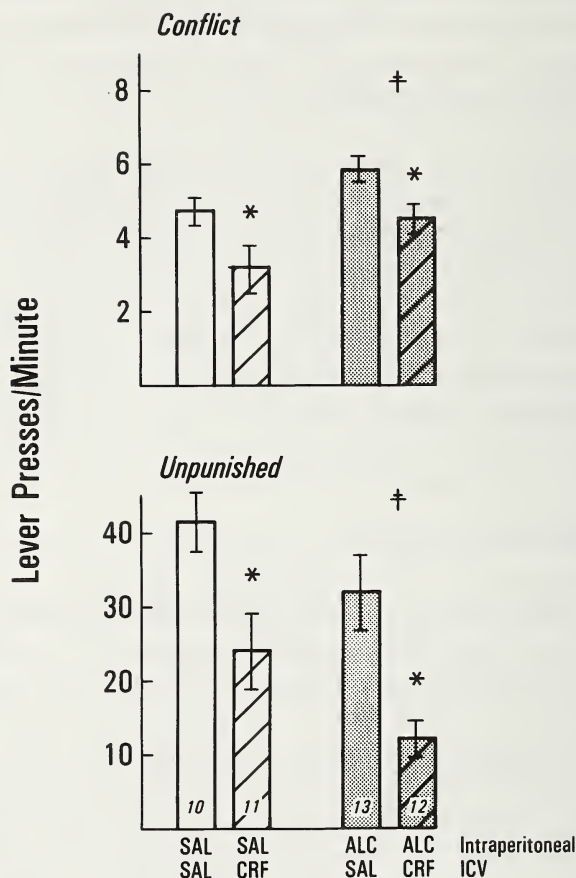


Figure 4.—Interaction of alcohol (0.75 g/kg) IP and CRF (0.5 μ g) ICV on responsiveness during the conflict (*top*) and unpunished (*bottom*) components of an operant conflict test. Number of rats in each group is indicated at bottom of each column. Results are expressed as responses per minute (mean \pm standard error). *, Significantly different from ICV saline (main CRF effect, ANOVA). †, Significantly different from IP saline (main alcohol effect, ANOVA). Reprinted with permission from *Regulatory Peptides* 16:315-320, 1986. Britton, K.T., and Koob, G.F. Alcohol reverses the proconflict effect of corticotropin releasing factor. Copyright 1986 by Elsevier Science Publishers BV, Amsterdam, The Netherlands.

corticosteroids after acute ethanol exposure have also been reported (Ellis 1966; Kakihana and Moore 1976; Tabakoff et al. 1978; Knynch and Prohaska 1981). Recent studies by Rivier et al. (1984) have revealed that the increases in ACTH secretion seen after acute ethanol administration in rats is highly dependent on CRF production, as immunoneutralization of endogenous CRF was found to totally abolish the ethanol-induced release of ACTH. Chronic exposure to ethanol vapors has also been shown to cause a decrease in hypothalamic content of CRF (Rivier et al. 1984).

In a recent study, the activating effects of centrally administered CRF were studied in ethanol-exposed animals in an attempt to examine the possibility that brain CRF systems become activated during chronic ethanol exposure. Male Wistar rats were exposed to chronic ethanol vapors for 21 days and then challenged with ICV administration of CRF. Responses to CRF were tested during chronic exposure, 1.5 hr after removal of ethanol vapors, and 2 weeks after withdrawal of ethanol. A greater sensitivity to the locomotor-activating effects of CRF was found in ethanol-treated rats than in the controls during ethanol exposure and 90 min after removal of ethanol vapors but not 2 weeks after withdrawal (Ehlers and Chaplin 1987). These results support clinical findings of a reversible activation in the HPA axis in alcoholism. In addition, it appears that chronic exposure to ethanol can also modify central neuronal systems specifically responsive to the locomotor-activating effects of CRF.

Conclusions

These results describing neuronal activation, sympathetic activation, EEG arousal, general behavior activation, and stress-enhancing actions of CRF all suggest a possible role for CRF as a fundamental activating system. The functional significance of this system may have developed as a means for an organism to mobilize not only the pituitary-adrenal system but also the CNS in response to environmental challenge. Indeed, results in our laboratory suggest that treatment with CRF can improve performance and that this effect is dose and task related. Aversive situations appear much more sensitive to exogenous CRF, and preliminary results suggest that these aversive states may be sensitive to administration of a weak CRF antagonist.

Clearly, a hypothetical CNS activation system definitively linked to the pituitary-adrenal system that can improve behavior performance at low levels of output but attenuate behavioral performance at high levels of output would be of certain survival value. It is not difficult to imagine a possible role for such a system in clinical disorders such as anxiety, affective disorders, and other psychopathology. In addition, in certain behavioral tests, CRF and ethanol can have opposite effects. With chronic ethanol exposure, brain CRF systems may become chemically hyperactive, and these systems could hypothetically be responsible for many of the adverse effects of ethanol withdrawal particularly as well as aspects of ethanol tolerance.

References

- Aston-Jones, S.; Aston-Jones, G.; and Koob, G.F. Cocaine antagonizes anxiolytic effects of ethanol. *Psychopharmacology* 84:28-31, 1984.
- Berridge, C.W., and Dunn, A.J. A corticotropin-releasing factor antagonist reverses the stress-induced changes of exploratory behavior in mice. *Hormones Behav* 21:393-401, 1987.
- Britton, K.; Lee, G.; Vale, W.; Rivier, J.; and Koob, G.F. Corticotropin releasing factor antagonists block activating and "anxiogenic" actions of CRF in the rat. *Brain Res* 369:303-306, 1986.
- Britton, K.; Morgan, J.; Rivier, J.; Vale, W.; and Koob, G.F. Chlordiazepoxide attenuates CRF-induced response suppression in the conflict test. *Psychopharmacology* 86:170-174, 1985.
- Britton, K.T., and Koob, G.F. Alcohol reverses the proconflict effect of corticotropin releasing factor. *Regulatory Peptides* 16:315-320, 1986.
- Brown, M.R., and Fisher, L.A. Central nervous systems effects of corticotropin releasing factor in the dog. *Brain Res* 280:75-79, 1983.
- Brown, M.R.; Fisher, L.A.; Rivier, J.; Spiess, J.; Rivier, C.; and Vale, W. Corticotropin-releasing factor: Effects on the sympathetic nervous system and oxygen consumption. *Life Sci* 30:207-210, 1982a.

- Brown, M.R.; Fisher, L.A.; Spiess, J.; Rivier, J.; Rivier, C.; and Vale, W. Comparison of the biologic actions of corticotropin releasing factor and sauvagine. *Regulatory Peptides* 4:107-114, 1982b.
- Brown, M.R.; Fisher, L.A.; Spiess, J.; Rivier, C.; and Vale, W. Corticotropin-releasing factor: Actions on the sympathetic nervous system and metabolism. *Endocrinology* 111:928, 1982c.
- Burchfield, S. The stress responses: A new perspective. *Psychosomatic Med* 41:661-672, 1979.
- De Souza, E.B. Corticotropin releasing factor receptors in the rat central nervous system: Characterization and regional distribution. *J Neurosci* 7:88-100, 1987.
- De Wied, D. Behavioral effects of neuropeptides related to ACTH, MSH and B-LPH. *Ann NY Acad Sci* 297:263-274, 1977.
- Eaves, M.; Britton, K.T.; Rivier, J.; Vale, W.; and Koob, G.F. Effects of corticotropin releasing factor on locomotor activity in hypophysectomized rats. *Peptides* 6:923-926, 1985.
- Ehlers, C.L., and Chaplin, R.I. Chronic ethanol exposure potentiates the locomotor activating effects of corticotropin-releasing factor (CRF) in rats (RPT 00644). *Regulatory Peptides* 19:345-354, 1987.
- Ehlers, C.L.; Henriksen, S.J.; Wang, M.; Rivier, J.; Vale, W.; and Bloom, F.E. Corticotropin releasing factor produces increases in brain excitability and convulsive seizures in the rat. *Brain Res* 278:332-336, 1983.
- Ellis, F.W. Effect of ethanol on plasma corticosterone levels. *J Pharmacol Exp Ther* 153:121-127, 1966.
- Gispen, W.H.; Weigant, V.M.; Greven, H.H.; and De Wied, D. The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: Structure activity. *Life Sci* 17:645-655, 1975.

- Kakihana, R., and Moore, J.A. Circadian rhythm of corticosterone in mice. The effect of chronic consumption of alcohol. *Psychopharmacology* 46:301-305, 1976.
- Knynch, E.T., and Prohaska, J.R. Effect of chronic intoxication and naloxone on ethanol-induced increase in plasma corticosterone. *Life Sci* 28:1987-1994, 1981.
- Koob, G.F.; Swerdlow, N.; Seeligson, M.; Eaves, M.; Sutton, R.; Rivier, J.; and Vale, W. Effects of alpha-flupenthixol and naloxone on CRF-induced locomotor activation. *Neuroendocrinology* 39:459-464, 1984a.
- Koob, G.F., Thatcher-Britton, K.; Britton, D.; Roberts, D.C.S.; and Bloom, F.E. Destruction of the locus coeruleus or dorsal noradrenergic bundle does not alter release of punished responding by ethanol and chlordiazepoxide. *Physiol Behav* 33:479-485, 1984b.
- Krahn, D.D.; Gosnell, B.A.; Grace, M.; and Levine, A.S. CRF antagonist partially reverse CRF- and stress-induced effects on feeding. *Brain Res Bull* 17:285-289, 1986.
- Rivier, C.; Bruhn, T.; and Vale, W. Effect of ethanol on the hypothalamic-pituitary-adrenal axis in the rat: Role of corticotropin-releasing factor (CRF). *J Pharmacol Exp Ther* 229:127-131, 1984.
- Rivier, J.; Rivier, C.; and Vale, W. Synthetic competitive antagonists of corticotropin-releasing factor: Effect on ACTH secretion in the rat. *Science* 224:889-891, 1984.
- Segal, D.S.; Browne, R.G.; Arnsten, A.; Derrington, D.C.; Bloom, F.E.; Guillemin, R.; and Ling, N. Characteristics of beta-endorphin-induced behavior activation and immobilization. In: Usdin, E.; Bunney, W.E.; and Kline, N.; eds. *Endorphins in Mental Health Research*, London: MacMillan, 1979.
- Sutton, R.E.; Koob, G.F.; Le Moal, M.; Rivier, J.; and Vale, W. Corticotropin releasing factor (CRF) produces behavioral activation in rats. *Nature* (London) 297:331-333, 1982.

- Swerdlow, N.R.; Geyer, M.A.; Vale, W.W.; and Koob, G.F. Corticotropin releasing factor potentiates acoustic startle in rats: Blockade by chlordiazepoxide. *Psychopharmacology* 88:142- 152, 1986.
- Swerdlow, N.R., and Koob, G.F. Separate neural substrates of the locomotor-activating properties of amphetamine, caffeine and corticotropin releasing factor (CRF) in the rat. *Pharmacol Biochem Behav* 23:303-307, 1985.
- Tabakoff, B.; Jaffe, R.C.; and Ritzmann, R.F. Corticosterone concentrations in mice during ethanol drinking and withdrawal. *J Pharm Pharmacol* 30:371-374, 1978.
- Takahashi, L.K.; Kalin, N.H.; Vanden-Burgt, J.A.; and Sherman, J.E. Corticotropin-releasing factor modulates defensive withdrawal and exploratory behavior in rats. *Behav Neurosci* 103:648-654, 1988.
- Tazi, D.; Dantzer, R.; Le Moal, M.; Rivier, J.; Vale, W.; and Koob, G.F. A role for central nervous system corticotropin-releasing factor in stress-induced fighting in rats. *Regulatory Peptides* 18:1-62, 1987.
- Tazi, A.; Dantzer, R.; Mormede, R.; and Le Moal, M. Effects of post-trial administration of naloxone and beta-endorphin on shock-induced fighting in rats. *Behav Neural Biol* 39:192-202, 1983.
- Tazi, A.; Dantzer, R.; Mormede, R.; and Le Moal, M. Effects of post-trial injection of beta-endorphin on shock-induced fighting are dependent on baseline of fighting. *Behav Neural Biol* 43:322-326, 1985.
- Vale, W.; Spiess, J.; Rivier, C.; and Rivier, J. Characterization of a 41 residue ovine hypothalamic peptide that stimulates the secretion of corticotropin and beta-endorphin. *Science* 213:1394-1397, 1981.
- Valentino, R.J.; Foote, S.L.; and Aston-Jones, G. Corticotropin-releasing factor activates noradrenergic neurons of the locus coeruleus. *Brain Res* 270:363-367, 1983.

Van Thiel, D. Adrenal response to ethanol: A stress response. In: Pohorecky, L.A., and Brick, J., eds. *Stress and Alcohol Use*. New York: Elsevier, 1983. pp. 23-27.

Neurotensinergic Processes: Potential Role In Ethanol Actions

*V. Gene Erwin*¹

Introduction

Innate differences in ethanol sensitivity, i.e., behavioral pharmacologic responses to ethanol, undoubtedly are related to why some individuals are at high risk while others are at low risk for alcoholism (Cotton 1979; Goodwin et al. 1973; Bohman et al. 1981; Schuckit 1980). Careful studies have shown that individuals at high risk for alcoholism differ from those at low risk in sensitivity to ethanol, e.g., subjective responses to ethanol and sensitivity to body sway (Schuckit 1984), and specific electroencephalogram patterns (Begleiter 1985; personal communication). In human (Wilson et al. 1983, 1984), and animal (Erwin et al. 1976) studies, it is well established that marked differences exist in sensitivity to ethanol. Characterization of the neuronal and neurochemical bases for individual differences in sensitivity to ethanol has been the subject of intensive research for the past decade. Recent studies suggest that differences in ethanol sensitivity may in part be mediated by neurotensinergic processes and that ethanol acts in part via neurotensin (NT) systems in the brain. NT is a tridecapeptide (p-Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) that was initially isolated from bovine hypothalamus (Carraway and Leeman 1976) and is widely distributed in the mammalian central nervous system (CNS) (Emson et al. 1982). Evidence strongly supporting a neurotransmitter role for NT

¹Alcohol Research Center, School of Pharmacy, University of Colorado, Boulder, CO 80309.

includes specific localization in discrete neuronal pathways, Ca^{2+} -dependent release from brain slices (Iversen et al. 1978), high-affinity specific binding sites in brain (Mazella et al. 1983; Uhl and Snyder 1977), and receptor-mediated neurochemical and behavioral actions.

Brain Neurotensin Localization

Immunohistochemical and radioimmunologic studies have shown the highest levels of NT to be located in the hypothalamus, midbrain, and striatum, including the nucleus accumbens (NA) (Emson et al. 1982; Cooper et al. 1981). Specific areas of the frontal cortex, the periaqueductal gray, and the olfactory tubercle are relatively high in NT immunoreactivity (NT-ir). These anatomic studies show three major neurotensinergic pathways (nigrostriatal, mesolimbic, and mesocortical) emanating from the substantia nigra and the ventral tegmental area (VTA), as well as hypothalamic neurons in the arcuate nucleus (Ibata et al. 1983; Jennes et al. 1982). Anatomic, pharmacologic, and neurochemical data indicate a functional role of NT in dopaminergic pathways (Nemeroff and Cain 1985; Fuxe et al. 1984). For example, evidence indicates that NT-ir found in the NA, caudate, and arcuate neurons may be colocalized with dopamine (DA) (Nemeroff et al. 1983). Numerous neuronal perikarya of the VTA contain NT-ir, and NT and DA are colocalized in some VTA neurons (Fuxe et al. 1984).

Neurotensin Receptors

NT-binding sites have been characterized in the CNS of mouse, rat, human, and other species (Mazella et al. 1983, 1988; Kanba et al. 1986). Recent studies have focused on characterization of two NT receptor subtypes in mouse and rat (Kitabgi et al. 1987; Schotte et al. 1986; Moyse et al. 1987). These receptors have been described as high-affinity (NT_2 or NT receptor) and low-affinity (NT_1 or NT acceptor) sites (Schotte et al. 1986) and have been distinguished by biphasic [^{125}I -Tyr³]- or [^{125}I -Trp³]-NT Scatchard analyses and by selectivity toward a nonpeptide histamine (H_1) antagonist, levocabastine (Janssen Pharmaceutica, Breese, Belgium). Levocabastine (1 μM) completely blocks NT binding to the NT_1 (low-affinity) receptor without affecting the NT_2 (high-affinity) receptor (Kitabgi et al. 1987; Schotte et al.

1986); this compound has been used as a simple method of distinguishing the two subtypes of NT receptors in autoradiographic (Szigethy and Beaudet 1987), subcellular localization (Schotte et al. 1988), and ontogenic (Schotte and Laduron 1987) studies. The identification of two subtypes of NT receptors has provided valuable information about the putative neurotransmitter function of NT. The levocabastine-sensitive NT receptors appear to be widely distributed throughout the brain, whereas levocabastine-insensitive NT receptors, which account for 10-20 percent of total NT binding, are less evenly distributed, with enriched populations in frontal cortex, corpus striatum, and midbrain (Kitabgi et al. 1987; Moyse et al. 1987). Recent studies (Schotte et al. 1988) using 6-OHDA lesions show that NT₂ (high-affinity sites) may be associated with presynaptic dopaminergic terminals in the striatum. Recent radiohistochemical studies indicate that NT receptors are closely associated with certain cholinergic neurons in the rat forebrain (Szigethy and Beaudet 1987) as well as with DA-containing neurons of the midbrain (Palacios and Kuhar 1981).

Characteristics of NT receptors on N1E-115 (a murine neuroblastoma cell line) cells have been well described (Gilbert and Richelson 1984; Amar et al. 1985), and these studies have demonstrated coupling of NT receptors to cyclic GMP formation. Recently, Richelson and co-workers (Gilbert and Richelson 1986; Kanba and Richelson 1987) have extended these studies to include structure-activity relationships for NT enhancement of both cyclic GMP formation and hydrolysis of inositol phospholipid, and other studies (Amar et al. 1987) have implicated GTP-binding protein in these actions of NT in N1E-115 cells. Current evidence suggests that NT receptors in brain slices and pituitary are coupled to production of inositol 1,4,5-trisphosphate (IP₃) by increasing inositol phospholipid hydrolysis (Goedert et al. 1984; Canonico et al. 1985). Such action would be expected to increase intracellular Ca²⁺ mobilization and enhance related events, including protein phosphorylation and neurosecretion. Likewise, ethanol has been shown to alter several processes associated with intracellular Ca²⁺ mobilization (Hood and Harris 1979; Harris and Fenner 1982). Only recently have the effects of ethanol on inositol phospholipid hydrolysis been reported (Gonzales et al. 1986), and Weiner et al. (1988) have recently shown that basal and norepinephrine-stimulated ³H-phosphatidylinositol (PI) turnovers are greater in SS than in LS mice.

Behavioral and Electrophysiologic Actions of Neurotensin

Several careful neuroanatomic studies have shown that behavioral responses to centrally administered NT are site selective (Kalivas et al. 1982*a,b*). Specifically, microinjection of NT into areas of the VTA that contain cell bodies of neurons projecting to the NA induced an increase in locomotor activity and in rearing similar to that observed by intra-NA administration of DA. Intra-NA injection of NT blocked the increase in activity associated with intra-VTA administration of NT (Quirion 1983). Whether the inhibition of locomotor activity due to intra-NA injection of NT is mediated via presynaptic receptors on terminals of neurons emanating from the VTA or via postsynaptic NT receptors on cell bodies in the NA is not well established. Other studies have provided evidence of site selectivity of NT effects in the CNS. For example, NT given intracerebroventricularly (ICV) was more potent in decreasing locomotor activity than it was by intra-NA administration and was most potent in producing hypothermia in the medial preoptic area, anterior hypothalamus, and VTA (Kalivas et al. 1982*b*).

When directly applied to cell bodies of neurons of the substantia nigra (Pinnock 1985; Pozza et al. 1988), preoptic-anterior hypothalamus (Baldino and Wolfson 1985), arcuate nucleus (Herbison et al. 1986), locus coeruleus (Olpe et al. 1987), or periaqueductal gray (Behbehani et al. 1987), NT produces depolarizing excitations consistent with receptor-mediated actions of the neuropeptide. Firing rate of rat cerebellar Purkinje neurons is inhibited by NT applied locally by microinjection, apparently due to increased release of norepinephrine from locus coeruleus-derived afferents (Marwaha et al. 1980). The latter observations are similar to those for the effects of ethanol on cerebellar Purkinje cells (Spuhler et al. 1982), where it has been shown that cerebellar Purkinje cells in LS mice are markedly more sensitive to the depressant effects of ethanol than are similar cells in SS mice.

NT-Ethanol Interactions

As summarized in table 1, acute ethanol administration produces many of the behavioral effects elicited by central NT administration. Likewise, there are similarities in the effects of ethanol and NT on dopaminergic systems. Acute

Table 1.—Comparisons of central actions of NT and ethanol

NT	Ethanol	NT-ethanol interaction
No anesthesia	Anesthesia	+
Hypothermia, ICV	Hypothermia	+
Motor inhibition, ICV	Motor inhibition (>2.0 g/kg)	+
Locomotor activation	Locomotor activation (<1.5 g/kg)	?
DA turnover in NA	DA turnover in NA	?
Hyperglycemia, ICV	Hyperglycemia	?
Analgesia, ICV	Analgesia	?
Self-administration (intra-VTA)	Self-administration (oral)	?
Tolerance	Tolerance	?

ethanol administration causes an increase in striatal DA turnover (Lai et al. 1979; Barbaccia et al. 1980), and Kalivas and Taylor (1985) reported that intra-VTA injections of NT increased DA turnover in the NA, an effect that was associated with an enhanced motor activity. The effects of ethanol on striatal dihydroxyphenyl acetic acid (DOPAC) levels were reported to depend on the genotype, with levels being elevated by ethanol in vivo in C57BL/6J but not in DBA/2J mice (Barbaccia et al. 1980). It is of interest that C57 mice are more sensitive than DBA mice to the sedative effects of ethanol (Spuhler et al. 1982). Recent studies reported that low doses of ethanol elicited an increased locomotor activity (Imperato and Di Chiara 1986) that was associated with an increase in DA release in the NA. A biphasic effect of ethanol was observed with depressant (high) doses producing a decrease in DA release.

Effects of Neurotensin on Ethanol-Induced Anesthesia

Studies with LS and SS mice have confirmed other reports that centrally administered NT enhances ethanol-induced anesthesia. Moreover, the studies (Erwin et al. 1987) showed that the action of NT was dependent on the mouse genotype. NT differentially altered ethanol-induced anesthesia, as measured by duration of loss of righting response (LRR) or by blood ethanol levels producing LRR in SS but not LS mice. This effect of NT was dose dependent, with doses of 5-500 ng ICV increasing ethanol sensitivity in SS but not LS mice. At higher doses, 0.5-10 μ g ICV, NT enhanced the sensitivity of both SS and LS mice to ethanol-induced anesthesia. Genetic selection of LS and SS mice was bidirectional, with ethanol sensitivity of these lines diverging from the sensitivity of the genetically heterogeneous foundation stock (HS). HS mice are intermediate between LS and SS mice in ethanol sensitivity. NT at 1.25 μ g ICV reduced the blood ethanol concentration (BEC) at LRR in both HS and SS mice to virtually the LS level. These results indicate that genetic differences in ethanol sensitivity in LS and SS mice might involve mechanisms associated with central NT processes. Another possibility is that activation of similar NT systems specifically alters signal transduction mechanisms that might differ genetically in LS and SS mice. The altered anesthetic sensitivity was specific for ethanol in that NT did not alter pentobarbital- or halothane-induced sleep time in either LS or SS mice.

The ability of NT to enhance ethanol-induced anesthesia in SS mice was specific for the carboxy-terminal fragment of NT. The NT analog *N*-acetyl-NT₈₋₁₃, but not NT₁₋₈, enhanced the anesthetic action of ethanol in SS mice. Results with NT analogs are in keeping with those of structural activity studies showing that NT₈₋₁₃, but not NT₁₋₈, binds to NT receptors and elicits NT-like responses. *N*-acetyl-NT₈₋₁₃ was less effective at 5 μ g ICV than was NT in decreasing the BEC at LRR, but at this dose it was equivalent to NT in enhancing ethanol-induced hypothermia. In the absence of ethanol, NT-induced hypothermia was virtually identical in LS and SS mice, suggesting that NT-mediated processes involved in thermoregulation are similar in these mouse lines. These results suggest that the effect of NT on ethanol sensitivity is the result of specific rather than nonspecific mechanisms and are consistent with the possibility that more than one NT receptor or receptor-mediated

process is involved in the potentiation of anesthetic and hypothermic actions of ethanol.

The potentiation of ethanol anesthesia was relatively unique for NT in that a number of neuropeptides were without effect in either LS or SS mice (Erwin et al. 1987). For example, bombesin, substance P, corticotropin-releasing factor, cholecystokinin, or somatostatin analogs at doses of 0.2 or 1.0 μg ICV did not markedly alter ethanol sensitivity. Although thyrotropin-releasing hormone (TRH) was not used in these studies, other investigators have reported that TRH decreased CNS sensitivity to the sedative and hypothermic effects of ethanol. More recently, Masserano and Weiner (1982) showed that this neuropeptide decreased ethanol sleep time in both SS and LS mice. β -Endorphin (β -END) was not as potent as NT at low doses, e.g., 0.2 μg . β -END was without effect on ethanol sensitivity; however, at 0.5 and 1.0 μg ICV, β -END was slightly more potent than NT in enhancing ethanol-induced anesthesia in SS mice. Differences in NT and β -END doses required to increase ethanol sensitivity could be due to differences not only in sites of action but also in rates of inactivation by peptidases in the brain, in affinity for the respective receptors, or in site accessibility after ICV injection.

The possibility that differences in the effects of NT on ethanol sensitivity might have been due to differences in metabolism of the neuropeptide in LS and SS brains was examined (Erwin et al. 1987). The data clearly show that the overall rates of degradation as measured by disappearance of [^3H]NT after ICV injection were similar for LS and SS mice. There was an initial rapid disappearance of [^3H]NT from the brain extracts in both LS and SS mice. This was followed by a slower rate of [^3H]NT disappearance. Recovery of radioactivity in the brain remained fairly constant up to 15 min after ICV injection. However, the decrease in intact [^3H]NT content in the extracts as determined by high-performance liquid chromatography separation was taken to represent metabolism by the brain tissue. As indicated, there were no differences in the rate of [^3H]NT disappearance in LS and SS brains, and it can be assumed that the differential effect of NT on ethanol sensitivity in LS and SS mice is not due to differential NT metabolism. This conclusion is supported by the data on the effect of [D-Trp 11]NT on ethanol sensitivity of LS and SS mice. It appears that the $t_{1/2}$ for intact [^3H]NT is about 5 min; thus, the effect of this neuropeptide on ethanol sensitivity in SS mice lasts much longer than the $t_{1/2}$. This observation is consistent with the potential

involvement of a second messenger in changing the sensitivity to ethanol.

Neurotensin and Ethanol-induced Hypothermia and Locomotor Activity

It is well known that ethanol produces hypothermia and that it produces a greater dose-dependent hypothermia in LS than in SS mice, with significant decreases in rectal temperature observed only at doses of greater than 3 g/kg given intraperitoneally. The hypothermic effect of ethanol determined at LRR was not altered in either LS or SS mice at low doses of NT, but at higher doses NT enhanced ethanol-induced hypothermia in both lines of mice (Erwin et al. 1987; Erwin and Su 1989). NT administered alone induced a similar hypothermia in both SS and LS mice at doses of greater than 0.02 μ g. However, doses of ethanol (1.0 g/kg) or NT (0.005 μ g ICV) that failed to cause hypothermia when administered separately produced a pronounced hypothermia when administered together (Erwin and Su 1989). Potentiation of NT- and ethanol-induced hypothermia was greater in SS than in LS mice. As noted by other investigators (Kalivas et al. 1982*a,b*), we observed that sensitivity to NT-induced hypothermia was greater after ICV administration than by infusion into the NA or VTA (Erwin and Su 1989).

It is well known that NT effects on spontaneous locomotor activity are brain region specific (Kalivas et al. 1982*a,b*), with increases or decreases in activity observed after intra-VTA or ICV injection, respectively. NT given ICV or intra-NA markedly inhibits ethanol-induced increase in locomotor activity in both SS and LS mice. The sites of NT action in decreasing locomotor activity after ICV administration are now known, but apparently these sites do not differ between LS and SS mice, since similar dose responses for NT-induced hypothermia were observed in these mice. NT administered intra-VTA produced a marked increase in locomotor activity in both LS and SS mice, an effect slightly more pronounced in SS than in LS animals. However, NT administered intra-VTA did not alter the effects of ethanol on locomotor activity.

The observations described above suggest that NT and ethanol may act in a synergistic manner on specific neuronal processes mediating thermoregulation and spontaneous motor activity. It is of interest that ICV doses of

NT required to elicit hypothermia were greater (50 ng) than those needed to alter locomotor activity (5 ng). These results indicate that NT may act on separate neuronal pathways in altering thermoregulation and locomotor activity.

Evidence indicating that exogenous administration of NT produces specific pharmacologic interactions with ethanol by actions through NT receptors provides a testable hypothesis that differences in acute sensitivity to ethanol are, in part, the result of genetic differences in specific NT-mediated processes. Potential sites of interaction of ethanol with neurotensinergic systems include (1) NT levels and turnover (synthesis, release, and degradation) in neurons projecting to the frontal cortex, striatum, NA, and hypothalamus; (2) NT receptors (pre- or postsynaptic, high or low affinity); (3) NT receptor-coupling processes, including second-messenger production; and (4) subsequent NT-mediated second-messenger responses (neurotransmitter release). Acute actions of ethanol could be altered by genetic differences in one or more of these neurotensinergic systems in discrete brain regions. For example, NT receptors in the NA and frontal cortex as well as receptors in the VTA and cerebellum are undoubtedly associated with behaviors characterized by motor functions, e.g., rearing and locomotor activity and perhaps the righting response, and hypothalamic receptors may be associated with thermoregulation. The hypothesis accommodates results of selective Ca^{2+} interactions on ethanol sensitivity in LS and SS mice (Morrow and Erwin 1986) in that most of these NT processes are Ca^{2+} dependent. Potentiating effects of coadministered Ca^{2+} and NT (Morrow and Erwin 1987) on ethanol sensitivity might suggest an ethanol-NT interaction with second-messenger systems coupled to increased intracellular Ca^{2+} . Indeed, Snider et al. (1986) have shown that NT increases not only IP_3 formation but also intracellular Ca^{2+} in N1E-115 cells.

Neurotensin Receptors in LS and SS Mice

The hypothesis that some of the acute effects of ethanol are mediated via neurotensinergic systems was investigated by characterizing NT receptors in LS and SS mice (Erwin and Korte 1988). [^3H]NT binding in brain membranes from both mouse lines was specific, saturable, reversible, and linear with protein concentrations. Subcellular localization studies showed

specific NT binding to be concentrated in the microsomal-synaptosomal fractions. Scatchard analyses of [^3H]NT binding indicated similar K_d values (ca.2-4 nM) for membranes from various brain regions of LS and SS mice. However, B_{max} values in frontal cortex, cerebellum, and striatum were greater in SS than in LS mice. In competitive binding studies, 50 percent inhibitory concentration (IC_{50}) values were lower for NT_{8-13} than for NT_{1-13} , and IC_{50} values for NT_{1-8} , NT_{1-11} , [D-Trp 11]NT, and [D-Tyr 11]NT were greater than 1,000 nM. Association and dissociation rate constants for [^3H]NT and resulting K_d values (0.8 nM) were similar for LS and SS brain membranes (Erwin and Korte 1988). Ethanol in vitro had no effect on NT-binding characteristics but, as expected, various cations markedly increased K_d values.

The studies summarized above were conducted to investigate the potential role of neurotensinergic systems in ethanol actions by examining characteristics of NT receptors and NT receptor-mediated behavior responses in LS and SS mice. The results show genetic differences in NT receptor densities in frontal cortex and striatum that might mediate the observed differences in NT-mediated behaviors between LS and SS mice and might be responsible in part for the genetically selected differences in ethanol-induced anesthesia in these mice. Since there is compelling evidence for the role of NT as a neurotransmitter, genetic variability in NT concentrations and receptors might be related to genetic differences in behavior and in pharmacologic responses to agents that act via neurotensinergic processes. The use of NT_{1-8} demonstrated the specificity of NT_{1-13} . In each case, the behaviors elicited by NT_{1-13} were not elicited by NT_{1-8} even at 10 times higher doses, indicating that the NT effects were specific receptor mediated. NT_{1-8} does not compete for NT_{1-13} in receptor assays, and it was without effect on locomotor activities or on hypnotic actions of ethanol in LS and SS mice.

The LS and SS mice were selectively bred from an HS population for differences in sensitivity to a hypnotic dose of ethanol. The LS and SS mice have subsequently been found to differ in a variety of responses to ethanol, including effects on locomotor activity and thermoregulation. Theoretically, the LS and SS mice should differ genetically only in those genes responsible for differences in sensitivity to ethanol; however, in 25 generations of selected breeding, significant fortuitous gene fixations probably have occurred. Thus,

it is somewhat speculative that genetic differences in NT receptors in specific brain regions account in part for differences in ethanol sensitivity. Nevertheless, it is tempting to speculate that some of the differences in behavioral effects of ethanol may be related to differences in NT receptor densities. This possibility is strengthened by the correlation between NT receptor density in the frontal cortex and ethanol sensitivity in LS, SS, and HS mice.

References

- Amar, S.; Kitabgi, P.; and Vincent, J.P. Stimulation of inositol phosphate production by neurotensin in neuroblastoma N1E115 cells; Implication of GTP-binding proteins and relationship with the cyclic GMP response. *J Neurochem* 49:999-1006, 1987.
- Amar, S.; Mazella, J.; Checler, F.; Kitabgi, P.; and Vincent, J.P. Regulation of cyclic GMP levels by neurotensin in neuroblastoma clone N1E115. *Biochem Biophys Res Commun* 129:117-125, 1985.
- Baldino, F., Jr., and Wolfson, B. Postsynaptic actions of neurotensin on preoptic-anterior hypothalamic neurons in vitro. *Brain Res* 325:161-170, 1985.
- Barbaccia, M.L.; Reggiani, A.; Spano, P.F.; and Trabucchi, M. Ethanol effects of dopaminergic function: Modulation by the endogenous opioid system. *Pharmacol Biochem Behav* 13(Suppl. 1):303-306, 1980.
- Begleiter, H. Symposium, AAAS, Los Angeles, CA, May 1985.
- Behbehani, M.M.; Shipley, M.T.; and McLean, J.H. Effect of neurotensin on neurons in the periaqueductal gray: An in vitro study. *J Neurosci* 7:2035-2040, 1987.
- Bohman, M.; Sigvardsson, S.; and Cloninger, C.R. Maternal inheritance of alcohol abuse: Cross-fostering analysis of adopted women. *Arch Gen Psychiatry* 38:965-969, 1981.

- Canonico, O.P.; Sortino, M.A.; Speciale, C.; and Scapagnini, U. Neurotensin stimulates polyphosphoinositide breakdown and prolactin release in anterior pituitary cells in culture. *Mol Cell Endocrinol* 42:215-220, 1985.
- Carraway, R., and Leeman, S.E. Radioimmunoassay for neurotensin, a hypothalamic peptide. *J Biol Chem* 251:7035-7044, 1976.
- Cooper, P.E.; Fernstrom, M.H.; Rorstad, O.P.; Leeman, S.E.; and Martin, J.B. The regional distribution of somatostatin, substance P and neurotensin in human brain. *Brain Res* 218:219-221, 1981.
- Cotton, N.S. The familial incidence of alcoholism: A review. *J Studies Alcohol* 40:80-116, 1979.
- Emson, P.C.; Goedert, M.; Horsfield, P.; Rioux, F.; and St. Pierre, S. The regional distribution and chromatographic characterization of neurotensin-like immunoreactivity in the rat central nervous system. *J Neurochem* 38:992-999, 1982.
- Erwin, V.G.; Heston, W.D.W.; and McClearn, G.E. Effects of hypnotics on mice genetically selected for sensitivity to ethanol. *Pharmacol Biochem Behav* 4:679-683, 1976.
- Erwin, V.G., and Korte, A. Brain neurotensin receptors in mice bred for differences in sensitivity to ethanol. *Alcohol* 5:198-201, 1988.
- Erwin, V.G.; Korte, A.; and Marty, M. Neurotensin selectively alters ethanol-induced anesthesia in LS/Ibg and SS/Ibg lines of mice. *Brain Res* 400:80-90, 1987.
- Erwin, V.G., and Su, N.C. Neurotensin and ethanol interactions on hypothermic and locomotor activity in LS and SS mice. *Alcoholism Clin Exp Res* 13:91-94, 1989.
- Fuxe, K.; Agnati, L.F.; Andersson, K.; Eneroth, P.; Härfstrand, A.; Goldstein, M.; and Zoli, M. Studies on neurotensin-catecholamine interactions in the hypothalamus and in the forebrain of the male rat. *Neurochem Interactions* 6:737-750, 1984.

- Gilbert, J.A., and Richelson, E. Neurotensin stimulates formation of cyclic GMP in murine neuroblastoma clone N1E-115. *Eur J Pharmacol* 99:245, 1984.
- Gilbert, J.A., and Richelson, E. LANT-6, xenopsin and neuromedin N stimulate cyclic GMP at neurotensin receptors. *Eur J Pharmacol* 129:379-383, 1986.
- Goedert, M.; Pinnock, R.D.; Downes, C.P.; Mantyh, P.W.; and Emson, P.C. Neurotensin stimulates inositol phospholipid hydrolysis in rat brain slices. *Brain Res* 323:193-197, 1984.
- Goodwin, D.W.; Schulsinger, F.; Hermansen, L.; Guze, S.B.; and Winokur, G. Alcohol problems in adoptees raised apart from alcoholic biological parents. *Arch Gen Psychiatry* 28:238-243, 1973.
- Gonzales, R.A.; Theiss, C.; and Crews, F.T. Effects of ethanol on stimulated inositol phospholipid hydrolysis in rat brain. *J Pharmacol Exp Ther* 237:92-98, 1986.
- Harris, R.A., and Fenner, D. Ethanol and synaptosomal calcium binding. *Biochem Pharmacol* 31:1790-1792, 1982.
- Herbison, A.E.; Hubbard, J.I.; and Sirett, N.E. Neurotensin excites neurons in the arcuate nucleus of the rat hypothalamus in vitro. *Brain Res* 364:391-395, 1986.
- Hood, W.F., and Harris, R.A. Effects of pentobarbital, ethanol and morphine on subcellular localization of calcium and magnesium. *Biochem Pharmacol* 28:3075-3080, 1979.
- Ibata, Y.; Fukui, K.; Okamura, H.; Kawakami, T.; Tanaka, M.; Obata, H.L.; Tsuto, T.; Terubayashi, H.; Yanaihara, C.; and Yanaihara, N. Co-existence of dopamine and neurotensin in hypothalamic arcuate and periventricular neurons. *Brain Res* 169:177-179, 1983.
- Imperato, A., and Di Chiara, G. Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. *J Pharmacol Exp Ther* 239:219-228, 1986.

- Iversen, L.L.; Iversen, S.D.; Bloom, F.; Douglas, C.; Brown, M.; and Vale, W. Calcium-dependent release of somatostatin and neurotensin from rat brain in vitro. *Nature* (London) 273:161-163, 1978.
- Jennes, L.; Stumpf, W.E.; and Kalivas, P.W. Neurotensin: Topographical distribution in rat brain by immunohistochemistry. *J Comp Neurol* 210:211-224, 1982.
- Kalivas, P.W.; Nemeroff, C.B.; and Prange, A.J., Jr. Neuroanatomical sites of action of neurotensin. *Ann NY Acad Sci* 400:397-418, 1982a.
- Kalivas, P.W.; Nemeroff, C.B.; and Prange, A.J., Jr. Neuroanatomical site specific modulation of spontaneous motor activity by neurotensin. *Eur J Pharmacol* 78:471-474, 1982b.
- Kalivas, P.W., and Taylor, S. Behavioral and neurochemical effect of daily injection with neurotensin into the ventral tegmental area. *Brain Res* 358:70-76, 1985.
- Kanba, K.S.; Kanba, S.; Okazaki, H.; and Richelson, E. Binding of [³H]-neurotensin in human brain: Properties and distribution. *J Neurochem* 46:946-952, 1986.
- Kanba, K.S., and Richelson, E. Comparison of the stimulation of inositol phospholipid hydrolysis and of cyclic GMP formation by neurotensin, some of its analogs, and neuromedin N in neuroblastoma clone N1E-115. *Biochem Pharmacol* 36:869-874, 1987.
- Kitabgi, P.; Rostène, W.; Dussaillant, M.; Schotte, A.; Laduron, P.M.; and Vincent, J.P. Two populations of neurotensin binding sites in murine brain: Discrimination by the antihistamine levocabastine reveals markedly different radioautographic distribution. *Eur J Pharmacol* 140:285-293, 1987.
- Lai, H.; Makans, W.L.; Horita, A.; and Leung, H. Effects of ethanol on turnover and function of striatal dopamine. *Psychopharmacology* 61:1-9, 1979.

- Marwaha, J.; Hoffer, B.; and Freedman, R. Electrophysiological actions of neurotensin in rat cerebellum. *Regulatory Peptides* 1:115-125, 1980.
- Masserano, J.M., and Weiner, N. Investigations into the neurochemical mechanisms mediating differences in ethanol sensitivity in two lines of mice. *J Pharmacol Exp Ther* 221:404-409, 1982.
- Mazella, J.; Chabry, J.; Kitabgi, P.; and Vincent, J.P. Solubilization and characterization of active neurotensin receptors from mouse brain. *J Biol Chem* 263:144-149, 1988.
- Mazella, J.; Poustis, C.; Labbe, C.; Checler, F.; Kitabgi, P.; Granier, C.; Van Reitschoten, J.; and Vincent, J.P. Monoiodo- [trp¹¹]-neurotensin, a highly radioactive ligand of neurotensin receptors. *J Bio Chem* 258:3476-3481, 1983.
- Morrow, E.L., and Erwin, V.G. Calcium influence on neuronal sensitivity to ethanol in selectively bred mouse lines. *Pharmacol Biochem Behav* 24:949-954, 1986.
- Morrow, E.L., and Erwin, V.G. Calcium influence on neurotensin and β -endorphin enhancement of ethanol sensitivity in selectively bred mouse lines. *Alcohol Drug Res* 7:225-232, 1987.
- Moyse, E.; Rostène, W.; Vial, M.; Leonard, K.; Mazella, J.; Kitabgi, P.; Vincent, J.P.; and Beaudet, A. Distribution of neurotensin binding sites in rat brain: A light microscopic radioautographic study using monoiodo [1²⁵I]Tyr₃-neurotensin. *Neuroscience* 22:525-536, 1987.
- Nemeroff, C.B., and Cain, S.T. Neurotensin-dopamine interactions in the CNS. *Trends Pharmacol Sci* 6:201-205, 1985.
- Nemeroff, C.B.; Luttinger, D.; Hernandez, D.E.; Mailman, R.B.; Mason, G.A.; Davis, S.D.; Widerlov, E.; Frye, G.D.; Kilts, C.A.; Beaumont, K.; Breese, G.R.; and Prange, A.J., Jr. Interactions of neurotensin with brain dopamine systems: Biochemical and behavioral studies. *J Pharmacol Exp Ther* 225:337-345, 1983.

- Olpe, H.R.; Steinmann, M.W.; Pozza, M.F.; and Haas, H.L. Comparative investigations on the actions of ACTH₁₋₂₄, somatostatin, neurotensin, substance P and vasopressin on locus coeruleus neuronal activity in vitro. *Naunyn-Schmiedeberg's Arch Pharmacol* 336:434-437, 1987.
- Palacios, J.M., and Kuhar, M.J. Neurotensin receptors are located on dopamine-containing neurons in rat midbrain. *Nature* (London) 294:587-589, 1981.
- Pinnock, R.D. Neurotensin depolarizes substantia nigra dopamine neurones. *Brain Res* 338:151-154, 1985.
- Pozza, M.F.; Kung, E.; Bischoff, S.; and Olpe, H.R. The neurotensin analog xenopsin excites nigral dopamine neurons. *Eur J Pharmacol* 145:341-343, 1988.
- Quirion, R. Interactions between neurotensin and dopamine in the brain: An overview. *Peptides* 4:609-615, 1983.
- Schotte, A., and Laduron, P.M. Different postnatal ontogeny of two [³H]neurotensin binding sites in rat brain. *Brain Res* 408:326-328, 1987.
- Schotte, A.; Leysen, J.E.; and Laduron, P.M. Evidence for a displaceable non-specific [³H]neurotensin binding site in rat brain. *Naunyn-Schmiedeberg's Arch Pharmacol* 333:400-405, 1986.
- Schotte, A.; Rostène, W.; and Laduron, P.M. Different subcellular localization of neurotensin-receptor and neurotensin-acceptor sites in the rat brain dopaminergic system. *J Neurochem* 50:1026-1031, 1988.
- Schuckit, M.A. Biological markers: Metabolism and acute reactions to alcohol in sons of alcoholics. *Pharmacol Biochem Behav* 13:9-16, 1980.
- Schuckit, M.A. Subjective responses to alcohol in sons of alcoholics and control subjects. *Arch Gen Psychiatry* 41:879-884, 1984.

- Snider, R.M.; Forray, C.; Pfenning, M.; and Richelson, E. Neurotensin stimulates inositol phospholipid metabolism and calcium mobilization in murine neuroblastoma clone N1E-115. *J Neurochem* 47:1214-1218, 1986.
- Spuhler, K.; Hoffer, B.; Weiner, N.; and Palmer, M. Evidence for genetic correlation of hypnotic effects and cerebellar Purkinje neuron depression in response to ethanol in mice. *Pharmacol Biochem Behav* 17:569-578, 1982.
- Szigethy, E., and Beaudet, A. Selective association of neurotensin receptors with cholinergic neurons in the rat basal forebrain. *Neurosci Lett* 83:47-52, 1987.
- Uhl, G.R., and Snyder, S.H. Neurotensin receptor binding, regional, and subcellular distribution favors transmitter role. *Eur J Pharmacol* 41:89-91, 1977.
- Weiner, E.A.; French, T.A.; and Masserano, J.M. Differential effects of norepinephrine on phosphoinositol turnover in the brains of long sleep (LS) and short sleep (SS) mice. *Pharmacologist* 30:A54, 1988.
- Wilson, J.R.; Erwin, V.G.; and McClearn, G.E. Effects of ethanol: I. Acute metabolic tolerance and ethnic differences. *Alcoholism Clin Exp Res* 8:226-232, 1983.
- Wilson, J.R.; Erwin, V.G.; McClearn, G.E.; Plomin, R.; Johnson, R.C.; Ahern, F.M.; and Cole, R.E. Effects of ethanol: II. Behavioral sensitivity and acute behavioral tolerance. *Alcoholism Clin Exp Res* 8:366-374, 1984.

Discussion

CAIN: I want to frame a few questions that I believe will be important to answer if we are to establish that peptides are important to alcohol effects or alcohol sensitivity. (1) Do specific peptides alter behavioral response to ethanol in sensitive versus nonsensitive animals? (2) Do animals that were bred to respond differentially to ethanol exhibit regionally selective differences in neuropeptide content, neuropeptide receptors, or neuropeptide

synthesis? From the talk by Dr. Erwin, you should have gotten a good idea of what needs to be done in this area. We need to be more specific in the brain regions that we are looking at. For example, instead of just looking at midbrain, dissect out specific mesencephalic nuclei for study. (3) Is there anatomical specificity to the neuromodulation of the effects of ethanol? Dr. Criswell has provided a very nice example of that in his exposition of the TRH antagonism of ethanol-induced sedation via an action in the medial septal nucleus. If one is looking at gross dissections or gross injections into the ventricle, one may miss important effects of neuropeptides in specific brain areas. (4) Do the neuropeptides modulate the effects of ethanol directly, or do they do it by interaction with other neurotransmitter or neuromodulator systems? We have talked about potential interaction of TRH with the gamma-aminobutyric acid (GABA) system. We haven't talked about arginine vasopressin-serotonin system today, but there is evidence that it may be involved in ethanol tolerance. Dr. Siggins talked about the acetylcholine-somatostatin interaction. We need to examine in more detail neuropeptides in conjunction with other neurotransmitters and neuromodulators. (5) Does ethanol alter signal transduction properties of neuropeptides, and are these effects anatomically selective? It has been mentioned, for example, that cGMP concentrations are influenced by ethanol administration. Neurotensin appears to be coupled to this second-messenger system. Perhaps, therefore, this coupling between NT and cGMP is altered by administration of alcohol. Another potential area is the interaction of TRH and the phosphatidylinositol hydrolysis-protein kinase C system. It might be interesting to look at that in the CNS and anterior pituitary with regard to ethanol intoxication. Another question that I have is whether or not in vitro studies with ethanol are equivalent to in vivo administration. When we give ethanol in vitro to a slice preparation or to a cell culture system, we lose the effects of other neurons that are important in vivo.

Finally, can neuropeptides be used clinically as therapeutic or diagnostic tools? The type of study that comes to mind is whether or not we can measure neuropeptides in the CSF of alcoholic patients. There are some preliminary data that β -endorphin is reduced in the CSF of chronic alcoholics. Another possibility is hormone stimulation tests. There is some evidence that following chronic ethanol administration there is a blunted TSH response to TRH and that CRF binding is altered in erythrocytes. It will be important to know if the differences in neuropeptides are present

before the ethanol is administered or are altered as a result of the presence of ethanol. It is important to know if the differences in neuropeptides are present before the ethanol is administered or are a result of the presence of ethanol.

ERIKSSON: I have a question for Dr. Erwin. In the SS and LS comparisons, you have the same problem that we have in the AT and ANT rats. That is, if you give a dose, which is effective in the SS mice to the LS mice, there is no more room for an effect, whereas if you would lower the dose given to the LS mice, an effect might be seen.

ERWIN: This is the horns of the dilemma. Do you give the same dose to both lines and have different pharmacologic responses, or do you give different doses to achieve the same pharmacologic response? One way around this is to give the ethanol orally, and then the blood alcohol rises slowly enough that you can measure the blood alcohol at the loss of the righting reflex. Unless there is differential development of tolerance in the 5-7 min that this requires, it will eliminate some of these concerns.

ERIKSSON: Another question for Dr. Koob is about the role of CRF. I wondered how you are distinguishing the effects of CRF from the effects of corticosteroids?

KOOB: We tested that hypothesis by giving dexamethasone or by using hypophysectomized or adrenalectomized animals. In those cases we can't see any difference; i.e., whenever we suppress the corticosterone, we still see the effects of CRF. While we haven't checked every behavioral test, we don't believe that the behavioral effects of CRF we see are due to corticosteroids.

GOLDSTEIN: I would like to use Dr. Erwin's paper to illustrate a concern I have had with the use of SS and LS mice in the decade that they have been generally available. You hope that the interaction of neurotensin and ethanol that you are examining is relevant to the primary hypnotic effect of ethanol. So it will be if both drugs work at the level of the major genes for which the LS and SS were originally separated. But the currently available LS and SS mice have changed in many ways over the years. We have discussed the accumulation of irrelevant genes, which gives us false-positive correlations. But I am also concerned with the accumulation of *relevant* genes. With

continued selection pressure, any genes that make LS slightly more sedatable and SS slightly more wide awake will have segregated, adding their effects to the differential response to ethanol. The lines, having now accumulated a variety of weak "sleepiness genes," will now differ in response to any sedative drug, obscuring the specificity for alcohols that was one of the most important findings of the LS/SS selection. What bothers me is the loss of an important advantage of selected lines in general, namely, that the animals can be assumed to have certain characteristics, established in early generations, and it should not be necessary to recheck these every time the lines are used. If it is necessary to go back and recheck the line differences for each new series of experiments, then little is gained over the use of HS mice.

ERWIN: I agree that it is a problem. Unfortunately, we can't go back and pick up earlier generations of LS and SS mice for testing. However, with the availability of recombinant inbred strains derived from a cross of SS and LS mice, we can begin to test whether or not these effects will hold up across these strains. This will allow us to look at correlations between ethanol-induced behavior and relevant neurochemistry.

VOGEL: If an animal is restrained, a lot of things happen. The heart rate goes up, blood pressure goes up, plasma steroids, amino acids, and catecholamines go up. If you pretreat with ethanol, you reduce or antagonize these effects. We get the same effect if we use any anxiolytic, diazepam or other benzodiazepines. In each case, they act almost identically to alcohol. I would like to ask if you see similar effects with the neuropeptides and anxiolytics as you see with alcohol?

KOOB: Yes and no. On the conflict component, yes. If we look at the unpunished component, then the answer is no. If we are looking at the unpunished response to CRF, ethanol potentiates the motor-disrupting effects of CRF response and makes the animals more ataxic.

VOGEL: In the first case you have an anxiolytic action, and in the second case you have more of a sedative action. So if we would only look at the anxiolytic action, then they would look the same.

DEITRICH: One comment to Dr. Goldstein's point is that in addition to the RI strains, we also now have the inbred SS and LS mice, which provide a

snapshot of what these animals were when this was accomplished. Another question to Dr. Criswell is whether TRH has an effect of the initial response to ethanol. Is there an effect of TRH on the ED₅₀ or on the blood alcohol at the loss of righting response?

CRISWELL: We don't have data on the effect of ethanol on the loss of righting reflex. Another comment about Dr. Vogel's question. In our case, TRH and the benzodiazepines give results similar to those obtained with ethanol and TRH, but if one gives a specific antagonist to the benzodiazepines, they don't do anything to the TRH response.

KOOB: First of all, at the doses of alcohol and chlordiazepoxide that we use, one does not see much analgesia. CRF at the doses that we use does not produce a hyperanalgesia effect. Also, in nonshock tests one still gets what can be interpreted as release of punished responding, such as in a simple open-field test where the animals have never been exposed to bright light. Ethanol will increase their tendency to go into the bright light.

VERNADAKIS: A question for Dr. Loh. The cells that you are using, the neuroblastoma, is a hybrid line. Do you feel comfortable in using these cells? I would also like to use them and would like your opinion on them. The criticism is usually that since these cells are not normal neurons, how can I interpret anything that I get with them.

LOH: It depends upon what you are looking for. I justify my work since I am interested in the opiate receptors and these cells have opiate receptors. However, when I finish I will not tell myself that this is the structure of the opiate receptor in the *brain*. As long as you know the limitations, then it is OK. They respond to morphine in a predictable way and it is a model system!

ALKANA: A comment for Dr. Criswell. When using drugs in attempts to identify the neurochemical events underlying initial sensitivity to ethanol, it is important to distinguish between drugs that antagonize ethanol by blocking or reversing it at a mechanistic site of action from other kinds of antagonism that might occur. In this context, your work suggests that TRH is a physiologic antagonist to ethanol. That is, it does not block or reverse ethanol at a molecular site of action. Rather, TRH offsets ethanol's effects

by inducing a physiologic response in the direction opposite that caused by ethanol. Therefore, manipulation of TRH can modify initial sensitivity to alcohol, but the system does not appear to represent a mechanistic site of action of alcohol.

CRISWELL: The term "site of action" has been used here, and it means different things to different people. In one case, the microinjection of neuropeptides into the brain identifies the site of action of these compounds in their interaction with ethanol to an accuracy of a few hundred microns. On the other hand, the site of action at a molecular level where a few angstroms are is quite a different thing.

SPUHLER: In Dr. Erwin's data, it appeared that the phenotypic variance that he presented for the HS population, an outbred stock, was no greater than for the SS and LS that now would be inbred with respect to the genes responsible for their differential response to ethanol. I wonder how that can be explained.

ERWIN: I can't explain it. Perhaps much of the variance is environmental variance and the part that is genetic variance may not contribute enough to significantly alter the overall variance.

GABA-Benzodiazepine Receptors: Chloride Channels

Structure of the γ -Aminobutyric Acid-Benzodiazepine Receptor Protein Complex: Focus on Central Nervous System Depressant Drug Action

R. W. Olsen, L. Deng, K.-Y. Huh, G. Smith, and M. Bureau¹

Introduction

The major inhibitory neurotransmitter γ -aminobutyric acid (GABA) acts principally via a postsynaptic membrane receptor (GABA_A) that also functions as a chloride ion channel (Olsen and Venter 1986; Biggio and Costa 1988). The chloride ion channel is opened by the binding of the neurotransmitter ligand, GABA, and this inhibits electrical activity of the postsynaptic cell (Yang and Olsen 1987; Barker et al. 1984; Macdonald et al. 1988). Consistent with their ability to modulate postsynaptic responses to GABA at the cellular level, numerous central nervous system (CNS) depressant and excitatory drugs have their sites of action right on the GABA receptor-chloride ion channel protein complex (Olsen 1981; Ticku and Maksay 1983; Olsen et al. 1988a). The benzodiazepines, the picrotoxinlike convulsants, and the barbiturates all have receptor sites on this GABA receptor complex (figure 1). Available evidence suggests that other general

¹Department of Pharmacology, School of Medicine, and Brain Research Institute, University of California, Los Angeles, CA 90024.

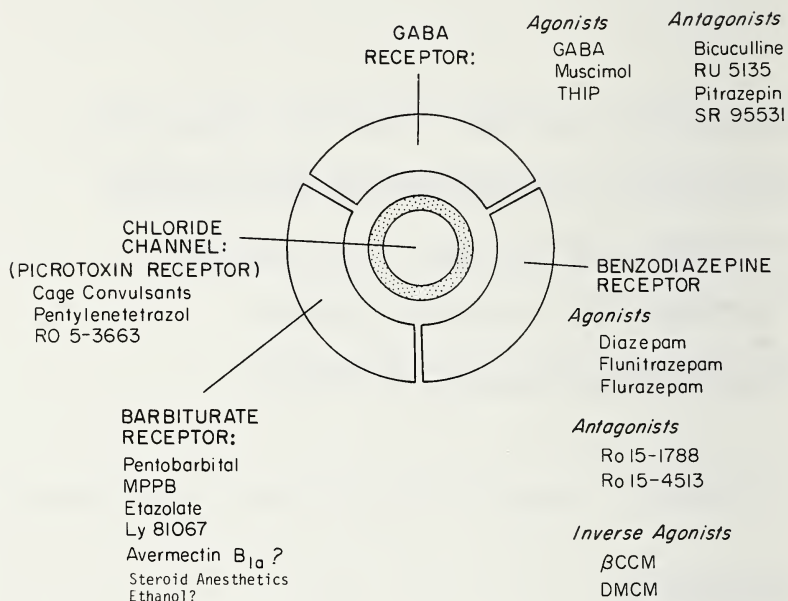


Figure 1.—Schematic model of the GABA receptor-chloride ion channel complex. Each of the sectors represents a distinct drug binding site, with examples of ligands for each listed at the side. No suggestion of protein subunits is intended. (From Olsen et al. 1988a. Reprinted with permission. Copyright 1988 by Plenum Publishing Corp.)

anesthetics (Huidobro-Toro et al. 1987), especially steroid anesthetics (Majewska et al. 1986; Olsen et al. 1988b), and possibly ethanol (Suzdak et al. 1986; Huidobro-Toro et al. 1987) modulate GABA function and have receptor sites on the complex.

The four (or more) drug receptor sites on the GABA receptor complex all show mutual chloride-sensitive allosteric interactions, suggesting close coupling between them (figure 1) (Olsen 1981; Olsen et al. 1988b). This macromolecular complex with all of the interactive binding sites can be

solubilized and purified (Stephenson et al. 1982; Sigel et al. 1983; Sigel and Barnard 1984; Stauber et al. 1987; King et al. 1987). Antibodies produced against the receptor protein immunoprecipitate all of the activities (Schoch et al. 1985; Stephenson et al. 1986; Mamalaki et al. 1987; Stauber et al. 1987; Vitorica et al. 1988). The complex has been cloned and the two peptide subunits have been sequenced, demonstrating that the two subunits are homologous both to each other and to other ligand-gated ion channels (Schofield et al. 1987). Furthermore, expression of GABA-regulated, drug-modulated chloride channel activity in frog oocytes, using the genetically engineered mRNA for these two peptides, indicates that they are necessary and sufficient for GABA receptor-chloride channel activity.

Evidence for CNS Depressant Drug Modulation of the GABA Receptor Complex

Barbiturates and related depressant agents such as etazolate, etomidate, and LY81067 (Ticku and Olsen 1978; Ticku and Maksay 1983; Olsen et al. 1988a) inhibit the binding of picrotoxinlike convulsant ligands for the chloride channel part of the GABA receptor complex in brain membranes (Olsen 1981). The same compounds allosterically enhance the binding of benzodiazepine receptor agonists and the binding of GABA receptor agonists *in vitro*; on the other hand, the binding activities of benzodiazepine inverse agonists and GABA receptor antagonists are allosterically inhibited by the barbiturates (Olsen et al. 1986). This finding is consistent with the action of barbiturates to potentiate the postsynaptic response to GABA by prolonging the open time of the chloride channel when GABA binds to the receptor (Barker et al. 1984; Biggio and Costa 1988). Thus, barbiturate binding favors the open-channel conformational state of the GABA receptor protein complex.

The barbiturates do not show sufficiently high affinity for direct binding assays; they are active only at concentrations of 10 μ M or higher. However, the interactions of the barbiturates with the other three receptor binding sites on the complex (GABA, benzodiazepine, and convulsant) serve to define a barbiturate receptor. The relative potencies of a long series of barbiturates and nonbarbiturates, including stereoisomers, correlate well for the *in vitro* interactions with GABA receptor complex binding and the biologic activity to

potentiate GABA function measured by either electrophysiologic techniques or $^{36}\text{Cl}^-$ flux measurements (Olsen 1981; Ticku and Maksay 1983; Olsen et al. 1986). Furthermore, this GABA interaction correlates with the *in vivo* activity as sedatives-hypnotics, suggesting that the pharmacologic activity involves enhancement of GABA-mediated inhibition in the CNS. Similar actions of steroid anesthetics (Majewska et al. 1986), other general anesthetics (Huidobro-Toro et al. 1987), and ethanol (Suzdak et al. 1986) support the notion that these CNS depressants act via enhancement of GABA-mediated inhibition (Biggio and Costa 1988).

The GABA and benzodiazepine receptor binding activities in brain membranes can be cosolubilized and copurified (Chang and Barnard 1982; Gavish and Snyder 1981; Stephenson et al. 1982; Sweetnam et al. 1987). Although picrotoxin-convulsant binding activity and barbiturate enhancement of GABA and benzodiazepine binding were not reproducibly demonstrable for the receptor solubilized in deoxycholate or Triton X-100, these activities were obtained upon solubilization with the zwitterionic bile salt detergent CHAPS (Stephenson and Olsen 1982; King and Olsen 1984). Addition of natural phospholipids to the detergent extract was also shown to favor "native" properties in the solubilized receptor (Bristow and Martin 1987). Consistent with the observations in membrane (Squires et al. 1983; Supavilai and Karobath 1984; Ticku and Maksay 1983), the "convulsant-barbiturate" activities were demonstrated to be associated with the GABA-benzodiazepine receptor complex and to copurify as a single protein (Sigel and Barnard 1984; King et al. 1987).

Biochemical Structure of the Drug-Modulated GABA Receptor-Chloride Channel Protein Complex

Table 1 summarizes a historical look at the known biochemical properties of the GABA receptor protein. The primary benzodiazepine binding site was identified as a 51-kilodalton (kD) peptide subunit by photoaffinity labeling with [^3H]flunitrazepam (Möhler et al. 1980). The apparent "native" molecular size (in detergent solution) of crude solubilized receptor was determined by sucrose gradient-gel filtration to be about 350 kDa (Stephenson et al. 1982) or 220 kDa (Chang and Barnard 1982). The latter

Table 1.—GABA-benzodiazepine receptor complex

Molecular weight (kDa)	Subunit(s)	Technique	Reference
220	51	Photoaffinity label	Möhler et al. 1980
		Target size	Chang and Barnard 1982
350		Size fractionation	Stephenson et al. 1982
220	(2x53) + (2x57)	Size fractionation and purification	Sigel et al. 1983
	51, 56	Monoclonal antibodies	Häring et al. 1985
> 400	55, 82	Target size	Nielsen et al. 1985
	51-53, 56-57	Photoaffinity label	Deng et al. 1986; Casalotti et al. 1986
400-500	31, 48, 52, 57	Purification	Stauber et al. 1987
		Size fractionation and target size	King et al. 1987
	53, 57	Cloning and expression	Schofield et al. 1987
	Multiple α and β	Photoaffinity labeling	Sieghart et al. 1983; Bureau and Olsen 1988 <i>a,b</i>
		In situ hybridization	Siegel 1988
	Multiple α	Cloning and expression	Levitan et al. 1988
	52 or 57	Photoaffinity labeling	Bureau and Olsen 1988 <i>a,b</i>
		Cloning and expression	Dionne and Barnard, unpublished data; Sakmann and Seeburg unpublished data
	57 only	Codfish	Deng et al. 1988

value was also consistent with irradiation target size analysis that gave identical 220-kDa estimates for both GABA and benzodiazepine binding activity (Chang and Barnard 1982). The purified bovine protein (Sigel et al. 1983; Sigel and Barnard 1984; Mamalaki et al. 1987) contained only two peptide bands in nearly equal amounts, suggesting an $\alpha_2\beta_2$ model ($2 \times 53 + 2 \times 56 = 218$ kDa). The suggestion that only these two peptide bands were integral receptor subunits was strengthened by the production of a family of monoclonal antibodies (Schoch et al. 1985) to the partially purified bovine receptor. These antibodies recognized either the 51- to 53-kDa α subunit or the 55- to 57-kDa β subunit (or neither) in purified or crude preparations (Häring et al. 1985). The 55- to 57-kDa subunit was identified as the primary GABA binding site by photoaffinity labeling with [^3H]muscimol (Deng et al. 1986; Casalotti et al. 1986).

The two subunits have been cloned and sequenced, and the genetically engineered mRNAs for these two peptides were injected into frog oocytes to express a drug-modulated, GABA-regulated chloride channel activity measured by electrophysiologic techniques (Schofield et al. 1987). This proves that these two peptides are necessary and sufficient for all activities of the complex (however, see below). The two subunits showed sequence homology with each other and with all subunits of the nicotinic acetylcholine receptor, indicating a gene superfamily for these ligand-gated ion channels. Both subunits contain four membrane-spanning domains, one or more of which make up the wall of the chloride channel. Consensus sequences are present in the extracellular N-terminal domain for glycosylation and in the cytoplasmic domain for phosphorylation (Schofield et al. 1987).

However, there remain some questions about the native molecular size and the subunit stoichiometry. Irradiation target size analysis showed molecular sizes of 220 kDa in one lab (Chang and Barnard 1982) but 50 kDa for both GABA and benzodiazepine binding in other labs (Schwartz et al. 1985; Nielsen et al. 1985) and 137 kDa for [^{35}S]t-butylbicyclopophosphorothionate (TBPS) binding to the convulsant site of the same protein, suggesting that more subunits are needed for this activity. Furthermore, the allosteric interactions between sites showed a target size of 350-500 kDa (Nielsen et al. 1985). The target size for solubilized benzodiazepine binding was about 100 kDa and that for [^{35}S]TBPS binding was about 300-500 kDa (King et al.

1987). Thus, the oligomeric size is between 200 and 500 kDa, and there may be more than four total subunits and more than two of each. An intriguing possibility would be five subunits, such as $\alpha_3\beta_2$ or $\alpha_2\beta_3$, in analogy to the nicotinic acetylcholine receptor (Boulter et al. 1987). Since the GABA and nicotinic receptor subunits show sequence homology, the oligomers may likewise contain a homologous number of subunits.

Subunit Subtypes

The subunit composition of an α and β oligomer is not yet definitive. In situ hybridization of the bovine α and β cDNA probes to mRNA in bovine cerebellum sections indicated that the two do not colocalize (Siegel 1988). This is probably because of a heterogeneity of the α or β gene product (or both) and possibly multiple genes for each. The original α and β may not be associated with each other in all cells in vivo, but with other partners. A microheterogeneity of benzodiazepine receptors has been implied by earlier photoaffinity labeling with [^3H]flunitrazepam. In addition to the major peptide band at 51 kDa, minor bands were observed in some brain areas, notably hippocampus, at 53, 55, and 59 kDa (Sieghart et al. 1983); furthermore, these bands were shown to have differential binding affinities for certain ligands such as the triazolopyridazines and beta-carbolines.

Additional related cDNA sequences for both cow and rat brain α and β subunits have been isolated by several groups (e.g., Khrestchatisky et al. 1988; Levitan et al. 1988). Three distinct bovine α cDNA sequences were isolated and expressed (along with β) in frog oocytes, indicating different dose dependences for GABA-activated chloride permeability (Levitan et al. 1988). All three species of channel were sensitive to barbiturates and picrotoxin, but none of the three (including the one previously reported by Schofield et al. [1987]) showed any benzodiazepine sensitivity of the GABA functional response (Levitan et al. 1988). The reason for this less than total biologic activity remains to be seen but could imply the need for additional subunits. Alternatively, the β subunit used in the expression studies might not normally be associated with these α subunits in vivo; therefore, the oligomers expressed in oocytes may not display the full native properties. A microheterogeneity of both α and β subunits on sodium dodecyl/sulfate gels has also been observed by protein staining, immunoblotting, and

photoaffinity labeling on purified receptor from cow, rat, and human brain (Olsen et al. 1988a; Bureau and Olsen 1988a).

Finally, oligomeric structures containing only one type of subunit must be considered, although both types of subunit are present in the isolated protein from bovine cortex (Sigel et al. 1983; Schofield et al. 1987), and the antibodies suggest that the two subunits occur together in all brain regions (Schoch et al. 1984; Häring et al. 1985; Mamalaki et al. 1987). The *in situ* hybridization studies (Siegel 1988) would be consistent with either hetero- or homo-oligomers. Photoaffinity labeling of the purified receptor indicated significant cross-labeling of the β subunits by flunitrazepam and of the α subunits by muscimol, suggesting that both major subunit types may contain binding sites for both types of ligand (Bureau and Olsen 1988b). The presence of GABA binding sites on α subunits has been confirmed by oocyte expression of GABA-activated chloride channels from a single mRNA, either α or β (Dionne and Barnard unpublished data; Sakmann and Seeburg unpublished data). Furthermore, we have identified the putative ancestral gene product in codfish brain, where the purified GABA-benzodiazepine receptor contains a single subunit of 57 kDa that is photoaffinity labeled by both [^3H]muscimol and [^3H]flunitrazepam (Deng et al. 1988).

Summary

The GABA receptor-chloride channel protein complex has been purified in large quantities, antibodies have been raised against it, and its two major subunits have been cloned, sequenced, and expressed. Multiple subtypes exist that vary in tissue distribution, developmental expression, and pharmacologic properties. Both individual variation and tissue variation in sensitivity to modulation by CNS depressants, including ethanol, are likely, and it will be very interesting to work out details of this very important neurobiology-neuropharmacology story.

References

Barker, J.L.; Gratz, E.; Owen, D.G.; and Study, R.E. Pharmacological effects of clinically important drugs on the excitability of cultured mouse spinal

- neurons. In: Bowery, N.G., ed. *Actions and Interactions of GABA and Benzodiazepines*. New York: Raven Press, 1984. pp. 203-216.
- Biggio, G., and Costa, E., eds. *Chloride Channels and Their Modulation by Neurotransmitters and Drugs*. New York: Raven Press, 1988.
- Boulter, J.; Connelly, J.; Deneris, E.; Goldman, D.; Heinemann, S.; and Patrick, J. Functional expression of two neuronal nicotinic acetylcholine receptors from cDNA clones identifies a gene family. *Proc Natl Acad Sci USA* 84:7763-7767, 1987.
- Bristow, D.R., and Martin, I.L. Solubilisation of the γ -aminobutyric acid/benzodiazepine receptor from rat cerebellum: Optimal preservation of the modulatory responses by natural brain lipids. *J Neurochem* 49:1386-1393, 1987.
- Bureau, M., and Olsen, R.W. Microheterogeneity of subunits in mammalian brain GABA/benzodiazepine receptor protein. *FASEB J* 2:A622, 1988a.
- Bureau, M., and Olsen, R.W. γ -Aminobutyric acid/benzodiazepine receptor protein carries binding sites for both ligands on both two major peptide subunits. *Biochem Biophys Res Commun* 153:1006-1011, 1988b.
- Casalotti, S.O.; Stephenson, F.A.; and Barnard, E.A. Separate subunits for agonist and benzodiazepine binding in the γ -aminobutyric acid_A receptor oligomer. *J Biol Chem* 261:15013-15016, 1986.
- Chang, L.-R., and Barnard, E.A. The benzodiazepine/GABA receptor complex: Molecular size in brain synaptic membranes and in solution. *J Neurochem* 39:1507-1518, 1982.
- Deng, L.; Olsen, R.W.; and Nielsen, M. [³H]muscimol and [³H]flunitrazepam photoaffinity label the same molecular weight band in codfish brain GABA/BZ receptor. *Abstr Soc Neurosci* 14:978, 1988.
- Deng, L.; Ransom, R.W.; and Olsen, R.W. [³H]muscimol photolabels the γ -aminobutyric acid receptor binding site on a peptide subunit distinct from

- that labeled with benzodiazepines. *Biochem Biophys Res Commun* 138:1308-1314, 1986.
- Gavish, M., and Snyder, S.H. γ -Aminobutyric acid and benzodiazepine receptors: Copurification and characterization. *Proc Natl Acad Sci USA* 78:1939-1942, 1981.
- Häring, P.; Stähli, C.; Schoch, P.; Takacs, B.; Staehelin, T.; and Möhler, H. Monoclonal antibodies reveal structural homogeneity of γ -aminobutyric acid/benzodiazepine receptors in different brain areas. *Proc Natl Acad Sci USA* 82:4837-4841, 1985.
- Huidobro-Toro, J.P.; Bleck, V.; Allan, A.M.; and Harris, R.A. Neurochemical actions of anesthetic drugs on the γ -aminobutyric acid receptor-chloride channel complex. *J Pharmacol Exp Ther* 242:963-969, 1987.
- Khrestchatsky, M.; MacLennan, A.J.; Olsen, R.W.; Bureau, M.; and Tobin, A.J. Isolation of cDNAs closely related to GABA-A receptor polypeptides. *Abstr Soc Neurosci* 14:978, 1988.
- King, R.G.; Nielsen, M.; Stauber, G.B.; and Olsen, R.W. Convulsant/barbiturate activities on the soluble GABA/benzodiazepine receptor complex. *Eur J Biochem* 169:555-562, 1987.
- King, R.G., and Olsen, R.W. Solubilization of convulsant/barbiturate binding activity on the GABA/benzodiazepine receptor complex. *Biochem Biophys Res Commun* 119:530-536, 1984.
- Levitan, E.S.; Schofield, P.R.; Burt, D.R.; Rhee, L.M.; Wisden, W.; Köhler, M.; Fujita, N.; Rodriguez, H.F.; Stephenson, A.; Darlison, M.G.; Barnard, E.A.; and Seeburg, P.H. Structural and functional basis for GABA_A receptor heterogeneity. *Nature (London)* 335:76-79, 1988.
- Macdonald, R.L.; Twyman, R.E.; Rogers, C.J.; and Weddell, M.G. Pentobarbital regulation of the kinetic properties of GABA receptor chloride channels. In: Biggio, G., and Costa, E., eds. *Chloride Channels*

- and Their Modulation by Neurotransmitters and Drugs*. New York: Raven Press, 1988. pp. 61-71.
- Majewska, M.D.; Harrison, N.L.; Schwartz, R.D.; Barker, J.L.; and Paul, S.M. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science* 232:1004-1007, 1986.
- Mamalaki, C.; Stephenson, F.A.; and Barnard, E.A. The GABA_A/benzodiazepine receptor is a heterotetramer of homologous α and β subunits. *EMBO J* 6:561-565, 1987.
- Möhler, H.; Battersby, M.K.; and Richards, J.G. Benzodiazepine receptor protein identified and visualized in brain tissue by a photoaffinity label. *Proc Natl Acad Sci USA* 77:1666-1670, 1980.
- Nielsen, M.; Honoré, T.; and Braestrup, C. Radiation inactivation of brain [³⁵S]butylbicyclophosphorothionate binding sites reveals complicated molecular arrangements of the GABA/benzodiazepine receptor chloride channel complex. *Biochem Pharmacol* 34:3633-3642, 1985.
- Olsen, R.W. GABA-benzodiazepine-barbiturate receptor interactions. *J Neurochem* 37:1-13, 1981.
- Olsen, R.W.; Bureau, M.; Ransom, R.W.; Deng, L.; Dilber, A.; Smith, G.; Khrestchatisky, M.; and Tobin, A.J. The GABA receptor-chloride ion channel protein complex. In: Kito, S.; Segawa, T.; Kuriyama, K.; Tohyama, M.; and Olsen, R.W., eds. *Neuroreceptors and Signal Transduction*. New York: Plenum Press, 1988a. pp. 1-14.
- Olsen, R.W., and Venter, J.C., eds. *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties*. Receptor Biochemistry and Methodology. Vol. 5. New York: Alan R. Liss, 1986.
- Olsen, R.W.; Yang, J.; King, R.G.; Dilber, A.; Stauber, G.B.; and Ransom, R.W. Barbiturate and benzodiazepine modulation of GABA receptor binding and function. *Life Sci* 39:1969-1976, 1986.

- Olsen, R.W.; Yang, J.S.-J.; and Ransom, R.W. GABA-stimulated $^{36}\text{Cl}^-$ flux in brain slices as an assay for modulation by CNS depressant drugs. In: Biggio, G., and Costa, E., eds. *Chloride Channels and Their Modulation by Neurotransmitters and Drugs*. New York: Raven Press, 1988b. pp. 125-133.
- Schoch, P.; Richard, J.G.; Häring, P.; Takacs, B.; Stähli, C.; Staehelin, T.; Haefely, W.; and Möhler, H. Co-localization of GABA_A receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies. *Nature* (London) 314:168-171, 1985.
- Schofield, P.R.; Darlison, M.G.; Fujita, N.; Burt, D.R.; Stephenson, F.A.; Rodriguez, H.; Rhee, L.M.; Ramachandran, J.; Reale, V.; Glencorse, T.A.; Seeburg, P.H.; and Barnard, E. Sequence and functional expression of the GABA_A receptor shows a ligand-gated receptor super-family. *Nature* (London) 328:221-227, 1987.
- Schwartz, R.D.; Thomas, J.W.; Kempner, E.S.; Skolnick, P.; and Paul, S.M. Radiation inactivation studies of the benzodiazepine/ γ -aminobutyric acid/chloride ionophore receptor complex. *J Neurochem* 45:108-115, 1985.
- Siegel, R.E. The mRNAs encoding GABA_A/benzodiazepine receptor subunits are localized in different cell populations of the bovine cerebellum. *Neuron* 1:579-584, 1988.
- Sieghart, W.; Mayer, A.; and Drexler, G. Properties of [^3H]flunitrazepam binding to different benzodiazepine binding proteins. *Eur J Pharmacol* 88:291-299, 1983.
- Sigel, E., and Barnard, E.A. A γ -aminobutyric acid/benzodiazepine receptor complex from bovine cerebral cortex. Improved purification with preservation of regulatory sites and their interactions. *J Biol Chem* 259:7219-7223, 1984.
- Sigel, E.; Stephenson, F.A.; Mamalaki, C.; and Barnard, E.A. A γ -aminobutyric acid/benzodiazepine receptor complex of bovine cerebral cortex: Purification and partial characterization. *J Biol Chem* 258:6965-6971, 1983.

- Squires, R.F.; Casida, J.E.; Richardson, M.; and Saederup, E. [³⁵S]-*t*-butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to γ -aminobutyric acid-A and ion recognition sites. *Mol Pharmacol* 23:326-336, 1983.
- Stauber, G.B.; Ransom, R.W.; Dilber, A.I.; and Olsen, R.W. The γ -aminobutyric acid-benzodiazepine receptor protein from rat brain: Large-scale purification and preparation of antibodies. *Eur J Biochem* 167:125-133, 1987.
- Stephenson, F.A.; Casalotti, S.O.; Mamalaki, C.; and Barnard, E.A. Antibodies recognizing the GABA_A/benzodiazepine receptor including its regulatory sites. *J Neurochem* 46:854-861, 1986.
- Stephenson, F.A., and Olsen, R.W. Solubilization by CHAPS detergent of barbiturate-enhanced benzodiazepine-GABA receptor complex. *J Neurochem* 39:1579-1586, 1982.
- Stephenson, F.A.; Watkins, A.E.; and Olsen, R.W. Physicochemical characterization of detergent-solubilized γ -aminobutyric acid and benzodiazepine receptor proteins from bovine brain. *Eur J Biochem* 123:291-298, 1982.
- Supavilai, P., and Karobath, M. [³⁵S]-*t*-butylbicyclophosphorothionate binding sites are constituents of the γ -aminobutyric acid-benzodiazepine receptor complex. *J Neurosci* 4:1193-1200, 1984.
- Suzdak, P.D.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. Ethanol stimulates γ -aminobutyric acid receptor-mediated chloride transport in rat brain synaptoneurosome. *Proc Natl Acad Sci USA* 83:4071-4075, 1986.
- Sweetnam, P.; Nestler, E.; Gallombardo, P.; Brown, S.; Duman, R.; Bracha, H.S.; and Tallman, J. Comparison of the molecular structure of GABA/benzodiazepine receptors purified from rat and human cerebellum. *Mol Brain Res* 2:223-233, 1987.

Ticku, M.K., and Maksay, G. Convulsant/depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complex. *Life Sci* 33:2363-2375, 1983.

Ticku, M.K., and Olsen, R.W. Interaction of barbiturates with dihydropicrotoxinin binding sites in mammalian brain. *Life Sci* 22:1643-1652, 1978.

Vitorica, J.; Park, D.; Chin, G.; and deBlas, A.L. Monoclonal antibodies and conventional antisera to the GABA_A receptor/benzodiazepine receptor/Cl⁻ channel complex. *J Neurosci* 8:615-622, 1988.

Yang, J., and Olsen, R.W. γ -Aminobutyric acid receptor regulated ³⁶Cl⁻ flux in mouse cortical slices. *J Pharmacol Exp Ther* 241:677-685, 1987.

Discussion

GOLDSTEIN: Is there some reason that one cannot get a high-specific-activity ligand for the barbiturate site?

OLSEN: The highest affinity ligand has a K_d of about 5 or 10 μ M. In order to get binding of such a molecule, one would have to have a specific activity much greater than 100 and a concentration of binding sites much greater than we have in brain. The only way that it could be done is with purified receptor in the test tube.

TABAKOFF: Also, one could not do a filtration assay given the low affinity of barbiturates for these binding sites.

BROWNING: Have you identified any sites that would be candidates for phosphorylation?

OLSEN: Yes. The first bovine beta subunit cloned by the Barnard group contains a consensus sequence for phosphorylation. However, no one has demonstrated that the GABA receptor is phosphorylated in vivo with any functional consequences. On the other hand, several neurophysiologic studies implicate regulation by phosphorylation.

Molecular Genetic Study of the Role of the γ -Aminobutyric Acid-Benzodiazepine Receptor in Neurosensitivity to Alcohol

James M. Sikela, W. Keir, and D. Wilson-Shaw¹

Introduction

Identification of specific genetic changes that influence sensitivity to alcohol may lead to an increased understanding of the molecular mechanisms underlying alcoholism. Toward this end, we have chosen to study the long-sleep (LS) and short-sleep (SS) mice, which have been bred for different sensitivities to alcohol (see Deitrich this volume). Given the fact that genetic differences exist in these animals which influence their sensitivity to alcohol, the question becomes, What are these genetic changes?

Recently, several groups have reported that the γ -aminobutyric acid (GABA)-benzodiazepine (BZ) receptor of LS mice appears to possess pharmacologic and biophysical properties different from those found in SS mice (Allan and Harris 1986; McIntyre et al. 1988; Marley et al. 1988). Although these changes may be due to differences in posttranslational modification(s) of the receptor between strains, they may also result from differences in the genes encoding the GABA-BZ receptor in the two strains. Therefore, as a first step toward determining whether the GABA-BZ receptor genes are different between LS and SS mice, we have isolated a cDNA clone corresponding to the α subunit of the mouse receptor.

¹Department of Pharmacology, School of Medicine, University of Colorado, Denver, CO 80262.

Results and Discussion

To isolate the mouse α -subunit cDNA, we synthesized an oligonucleotide (40-mer) corresponding to a region of the published bovine α -subunit cDNA sequence (Schofield et al. 1987) and used it as a probe to screen a mouse brain cDNA library. The oligonucleotide was derived from the 5'-most part of the protein-coding region of the bovine cDNA for two reasons: (1) protein-coding regions tend to be more conserved than noncoding regions between species and (2) probes derived from the 5' end of an mRNA would be more likely to identify full-length or near-full-length cDNAs. The mouse cDNA library (gift of Yoav Citri) was constructed from poly(A)⁺ mRNA derived from total brain of BALB/c mice. The cDNA was size selected for inserts of >2 kilobase pairs (kb) before cloning into the phage vector λ gt11 (Young and Davis 1983).

Approximately 5×10^5 recombinants from this library were screened by plaque hybridization (Benton and Davis 1977), using as a probe the bovine-derived oligonucleotide that had been end labeled with ^{32}P . A single positive clone containing a cDNA insert of approximately 2.6 kb was identified. Partial DNA sequencing of this cDNA (figure 1) indicated that it was very similar to the bovine α_1 cDNA and identical at the amino acid level to the bovine α_1 subunit. This finding is not surprising, given that the oligonucleotide used as a probe was derived from the signal sequence of the bovine α_1 subunit, a region that is not conserved between members of the α family (Levitan et al. 1988).

Because of the existence of multiple members of the α subunit family, we have initiated experiments to isolate other members of the α family in mouse. Using the α_1 mouse cDNA as a probe, we have screened the mouse brain library under low-stringency conditions and have isolated and plaque purified 26 additional cDNAs. Characterization of these clones should establish which members of the α subunit family are represented among these cDNAs.

The availability of cDNAs representing the different subunits of the mouse receptor will provide a useful resource for exploring possible sequence differences in the GABA-BZ receptor subunits between the LS and SS mice. The availability of the mouse α_1 cDNA also permits exploration of the presence of restriction fragment length polymorphisms (RFLPs) that are

b	ATG AAG AAA AGT CCG GGT CTC TCT GAC TAC CTT TGG GCC TGG ACC
b	CTC TTT CTG AGC ACA TTG ACT GGA AGA AGC TAT GGA CAA CCC TCA
m	CAA GAT GAA CTT AAA GAC AAC ACC ACT GTC TTC ACA AGA ATT
b	TTA CAA GAT GAA CTT AAA GAC AAC ACC ACT GTC TTC ACC AGG ATT
	* *
m	TTG GAC CGA CTG CTG GAC GGT TAT GAC AAT CGT CTG AGA CCA GGT
b	TTG GAC CGA CTC CTA GAT GGT TAT GAC AAT CGC CTG AGA CCG GGA
	* * *
m	TTG GGA GAG CGT GTA ACT GAA GTG AAG ACC GAC ATT TTC GTC ACC
b	TTA GGA GAG CGT GTA ACT GAA GTG AAG ACT GAC ATC TTC GTC ACC
	* *
m	AGT TTC GGA CCA GTT TCA GAC CAC GAT ATG GAG TAT ACA ATA GAT
b	AGT TTC GGA CCC GTT TCA GAC CAT GAT ATG GAA TAT ACA ATA GAT
	* *
m	GTG TTT TTC CGT CAA AGT TGG AAG GAT GAA AGA TTA AAA TTC
b	GTA TTT TTC CGT CAA AGC TGG AAG GAT GAA AGG TTA AAA TTT. . .
	* *

Figure 1.—Preliminary partial sequence alignment between the α_1 cDNA of bovine (b) and mouse (m). Underlined region represents the sequences used to design the oligonucleotide used to isolate the mouse cDNA. Differences between the two sequences are indicated (*).

associated with the α subunit gene(s) (Botstein et al. 1980). If present, such RFLPs could then be used for analysis of LS, SS, and recombinant inbred (RI) (DeFries et al. 1989) mouse strains to determine whether a qualitative correlation exists between the RFLP(s) and differential sensitivity to alcohol. The correlation would be possible only to the extent that RI strains that are SS-like phenotypically should have the SS RFLP and vice versa. Identification of a correlation between inheritance of a particular GABA-BZ receptor-associated RFLP allele and a particular sensitivity to alcohol would suggest that changes in the receptor gene influence sensitivity to alcohol.

References

- Allan, A.M., and Harris, R.A. Gamma-aminobutyric acid and alcohol actions: Neurochemical studies of long sleep and short sleep mice. *Life Sci* 39:2005-2015, 1986.
- Benton, W.D., and Davis, R.W. Screening λ gt recombinant clones by hybridization to single plaques in situ. *Science* 196:180-182, 1977.
- Botstein, D.; White, R.L.; Skolnick, M.; and Davis, R.W. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Human Genet* 32:314-331, 1980.
- Defries, J.C.; Wilson, J.R.; Erwin, V.G.; and Peterson, D.R. LSxSS recombinant inbred strains of mice: Initial characterization. *Alcoholism Clin Exp Res* 13:196-200, 1989.
- Levitan, E.S.; Schofield, P.R.; Burt, D.R.; Rhee, L.M.; Wisden, W.; Kohler, M.; Norihisa, F.; Rodriguez, H.F.; Stephenson, A.; Darlison, M.G.; Bernard, E.A.; and Seeburg, P.H. Structural and functional basis for GABA-A receptor heterogeneity. *Nature* (London) 335:76-79, 1988.
- Marley, R.J.; Stinchcomb, A.; and Wehner, J.M. Further characterization of benzodiazepine receptor differences in long-sleep and short-sleep mice. *Life Sci* 43:1223-1231, 1988.
- McIntyre, T.D.; Trullas, R.; Skolnick, P. Differences in the biophysical properties of the benzodiazepine/gamma-aminobutyric acid receptor chloride channel complex in the long-sleep and short-sleep mouse lines. *J Neurochem* 51:642-647, 1988.
- Schofield, P.R.; Darlison, M.G.; Fujita, N.; Burt, D.R.; Stephenson, F.A.; Rodriguez, H.; Rhee, L.M.; Ramachandran, V.R.; Glencorse, T.A.; Seeburg, P.H.; and Barnard, E.A. Sequence and functional expression of the GABA_A receptor shows a ligand-gated receptor super-family. *Nature* (London) 328:221-227, 1987.

Young, R.A., and Davis, R.W. Efficient isolation of genes by using antibody probes. *Proc Nat Acad of Sci* 80:1194-1198, 1983.

Discussion

PERIS: Why do you see the RFLP only in the RI and not in the LS or SS lines?

SIKELA: There are a number of explanations. One is that those differences occurred after the RI6 strain was initiated. Another is that the one LS mouse that we looked at was different from mice of the LS line that was used to start the RI strains. We need to look at a number of LS mice to see if it occurs in those animals, since the RI6 strain is more LS-like in the sleep time response to ethanol.

TABAKOFF: Did you use other restriction enzymes?

SIKELA: We used about 10 restriction enzymes, and *HindIII* is the only one to show a difference.

Brain Chloride Channels and Alcohol Action: Neurochemical Studies¹

R. Adron Harris and Andrea M. Allan²

Introduction

Rationale for Study of γ -Aminobutyric Acid (GABA)-activated Chloride Channels

To understand the mechanisms responsible for ethanol intoxication, tolerance, and dependence, we must identify ethanol's sites of actions in the brain. It is accepted that ethanol acts on brain membranes to alter some critical functions, but further progress requires identification of membrane proteins whose functions are affected by ethanol. We will set forth six criteria for identifying neurochemical functions that are likely responsible for the action of ethanol and briefly document that GABA-activated chloride channels fulfill these criteria. We maintain that this is the only membrane function that has thus far been shown to satisfy all criteria.

¹This study was supported by funds from the Veterans Administration and Public Health Service grants AA05233, AA06399, and AA03527.

²Denver VA Medical Center, Department of Pharmacology and Alcohol Research Center, University of Colorado School of Medicine, Denver, CO 80262.

(1) The membrane function should be altered by *in vitro* exposure to ethanol, with threshold effects observed at 5-20 mM, i.e., concentrations producing mild intoxication *in vivo*. Three different laboratories have shown that ethanol enhances GABA-activated $^{36}\text{Cl}^-$ flux at a threshold concentration of 5-15 mM (Allan and Harris 1987*a*; Allan and Harris 1986; Suzdak et al. 1986 *a,b*; Ticku and Kulkarni 1988).

(2) It should be possible to conceptually link this action of ethanol to ataxia, sedation, etc. GABA is the major inhibitory neurotransmitter in the central nervous system (CNS) and has important influences in cerebellum, cortex, and other brain regions. Augmentation of GABA action is likely to produce diverse depressant effects.

(3) Drugs that mimic the neurochemical action of ethanol should enhance behavioral actions of ethanol and/or act as intoxicants-anesthetics. Six different GABA-mimetic agents have been shown to enhance ethanol action (Frey and Breese 1982; Martz et al. 1983), and the GABA agonist THIP produces anesthesia alone. In addition, barbiturates, benzodiazepines, other alcohols, chloroform, and diethyl ether enhance GABA-activated chloride flux (Allan et al. 1988*a*; Huidobro-Toro et al. 1987), and these compounds all produce intoxication and anesthesia *in vivo*.

(4) Drugs producing neurochemical action opposite that of ethanol should reduce behavioral actions of ethanol. The GABA-activated chloride flux is reduced by bicuculline, picrotoxin, and benzodiazepine inverse agonists, and all of these drugs reduce at least some of the behavioral effects of ethanol (Frye and Breese 1982; Lister and Nutt 1987; Martz et al. 1983).

(5) Development of tolerance to ethanol should result in a change in function. Membranes from ethanol-tolerant animals display reduced sensitivity to the effects of ethanol (Allan and Harris 1987*a*; Morrow et al. 1988) and GABA (Morrow 1988) on chloride flux.

(6) The membrane function should be altered in animals selected for genetic differences in ethanol sensitivity. We studied the long-sleep/short-sleep (LS/SS), high-alcohol-sensitive (HAS)-1/low-alcohol-sensitive (LAS)-1, HAS-2/LAS-2, diazepam-sensitive/-resistant (DS/DR), and individual heterogeneous stock (HS) mice, and in all cases animals resistant to effects

of ethanol *in vivo* showed resistance to the effects of ethanol on GABA-activated chloride flux (Allan et al. 1988*a,b*; Allan and Harris 1986; Harris and Allan 1989). This genetic difference has been shown by using membranes from cortex (LS/SS, HAS/LAS, DS/DR, and HS), cerebellum (LS/SS and HAS/LAS), and whole brain (DS/DR) (Allan et al. 1988*a,b*; Allan and Harris 1986). These genetic differences in ethanol action are not merely quantitative but are marked and qualitative because the chloride channels of the ethanol-resistant animals do respond to ethanol at all. These results suggest major genetic differences in the structure or regulation of GABA-activated chloride channels.

However, we should point out that there are findings which are not consistent with the hypothesis that ethanol acts to enhance GABA-activated chloride conductance. (1) A number of electrophysiological studies (mainly using spinal neurons) indicate that ethanol enhances GABA action (Celentano et al. 1988; Davidoff 1973; Mereu and Gessa 1985; Nestoros 1980; Nishio and Narahashi 1988). However, other electrophysiologic studies have not found this action (e.g., Gage and Robertson 1985; Mancillas et al. 1986; Harrison et al. 1987). This raises questions about the generality and importance of the chloride flux studies of ethanol action. One possible explanation for these discrepancies is brain regional differences in ethanol response. For example, all of the negative studies used hippocampal neurons, and we also found that ethanol did not enhance chloride flux of hippocampal membranes (Allan and Harris 1986). We speculate that ethanol may not affect all GABA-activated chloride channels. However, at present it is not possible to define molecular changes which confer ethanol sensitivity or insensitivity on GABA-activated chloride channels. (2) The interactions between ethanol and benzodiazepine inverse agonists are complex and controversial, but it is clear that drugs such as Ro 15-4513 do not antagonize all actions of ethanol even though they reduce the effect of ethanol on chloride flux (Lister and Nutt 1987; Suzdak et al. 1986*a,b*; Sweetnam and Tallman 1985). Our data (Harris et al. *in press*) indicate that Ro 15-4513 can only partially antagonize effects of ethanol on chloride flux (although others find complete antagonism; Suzdak et al. 1986*a,b*; Ticku and Kulkarni 1988). The behavioral and neurochemical findings can be reconciled by proposing that not all GABA-activated chloride channels which are sensitive to ethanol are also sensitive to Ro 15-4513. (3) There is not a perfect correlation for genetic differences in ethanol sensitivity *in vivo* and *in vitro*. Membranes from animals selected for resistance to

ethanol display little or no effect of ethanol on chloride flux (see below), yet ethanol can produce intoxication and anesthesia in these animals if they are given sufficiently large doses. Alcohol sensitivity is known to be a polygenic trait (Dudek and Abbott 1984), and these results suggest that augmentation of chloride flux is only one of several mechanisms responsible for genetic differences in alcohol intoxication. However, we feel that it is the mechanism which is presently most amenable to detailed molecular analysis and that such an analysis will lead to a more complete model and perhaps answer the criticisms of the present model.

Structure and Function of the GABA-activated Chloride Channel

GENERAL

The channel complex contains receptors for at least four types of drugs: GABA agonists (GABA, muscimol, THIP, and isoguvacine) and antagonists (bicuculline and SR9551; benzodiazepines; convulsants (picrotoxin and TBPS); and barbiturates. GABA agonists activate the chloride channel, and this action is allosterically enhanced by benzodiazepine agonists (e.g., flunitrazepam) or barbiturates and allosterically inhibited by benzodiazepine inverse agonists (e.g., FG7142) and convulsants. The actions of all of these agents may be studied by analyzing uptake of ^{36}Cl by isolated brain microsacs (Allan et al. 1988*a,b*; Allan and Harris 1987*a,b*; Allan and Harris 1986).

MOLECULAR GENETICS

Molecular cloning has thus far identified two subunits of the channel complex, alpha and beta. There are multiple alpha subunits (three have been cloned and sequenced to date), but only one beta subunit has been cloned at this time (Levitan et al. 1988). It has been suggested that the channel complex is an $\alpha_2\beta_2$ tetramer and that the alpha subunit contains receptors for benzodiazepines while the beta subunit contains GABA receptors (Mamalaki et al. 1987; Schofield et al. 1987). However, this model may be incorrect. Recent studies show that both subunits contain the receptors for GABA and benzodiazepines (Bureau and Olsen 1988). Expression of cloned subunits (alpha, beta, or both) in *Xenopus* oocytes results in responses to GABA, pentobarbital, and picrotoxin, but benzodiazepines produce little

(Schofield et al. 1987) or no (Levitan et al. 1988) response. However, expression of brain poly(A)⁺ RNA (mRNA) results in robust benzodiazepine responses in oocytes (Houmed et al. 1984; Sigel and Baur 1988*a,b*), suggesting that additional receptor subunits, modulators, or posttranslational modifications are required for benzodiazepine responses. A recent abstract suggests that a gamma subunit exists and that this subunit is required for benzodiazepine sensitivity (Pritchett et al. 1988). There is also evidence that not all of the GABA-activated chloride channels of brain are sensitive to benzodiazepines (Supavilai et al. 1986), suggesting channel (and receptor) heterogeneity which may result in ethanol-sensitive and ethanol-insensitive channels.

POSTTRANSLATIONAL MODULATION

The key question here is whether function of the GABA-activated chloride channel may be modulated by posttranslational modifications such as phosphorylation or by intracellular modulators such as calcium or GTP. Such modulation may be responsible for the actions of ethanol on this channel and for genetic differences in channel function.

The ability of GABA to activate chloride channels is clearly suppressed by intracellular calcium concentrations that occur in stimulated cells (ca. 1 μ M) (Inoue et al. 1986; Stelzer et al. 1988; Taleb et al. 1987). It is not known whether ethanol, barbiturate, or benzodiazepine responses are dependent on intracellular calcium. Because ethanol increases intracellular calcium, it is important to determine whether this action reduces the effect of ethanol on chloride flux. We and others observed that the maximum effect of ethanol on chloride flux occurs at 10-30 mM, with 50-100 mM often producing less augmentation of flux; this finding is consistent with the hypothesis that the effects of ethanol on intracellular calcium inhibit its action on chloride flux.

The beta subunit of the chloride channel complex has a sequence appropriate for phosphorylation (Schofield et al. 1987), and the homologous nicotinic cholinergic receptor-channel complex appears to be regulated by phosphorylation (Huganir et al. 1988). Recent studies suggest that activation of protein kinase C or cAMP-dependent kinase inhibits activation of the chloride channel by GABA (Harrison and Lambert 1989; Sigel and Baur 1988*a,b*). In contrast to these results, "phosphorylation factors" (e.g., Mg-

ATP) are necessary to maintain the GABA response of cultured neurons (Stelzer et al. 1988). It is not known whether these treatments lead to phosphorylation of the channel complex or if they are indirect modulators of function. It is also not known whether kinase activity alters the actions of ethanol, barbiturates, or benzodiazepines on chloride flux, but our preliminary studies with cholera toxin suggest such a modulation. Partially purified preparations of the GABA receptor-channel complex contain a protein kinase activity that phosphorylates the alpha subunit of the complex (Suzdak et al. 1986). However, this kinase is not activated by known second messengers (e.g., cAMP, calcium, calmodulin, and phosphatidylserine) or by drugs acting on the GABA, benzodiazepine, or convulsant sites of the complex (Suzdak et al. 1986*a,b*). Thus, the functional importance of this alpha subunit phosphorylation remains unknown.

The chloride channel complex is extensively glycosylated, but the functional significance of glycosylation is not well understood. Deglycosylation of the complex with endoglycosidase H, which cleaves the carbohydrate complex near the protein, increased the B_{\max} of binding of a benzodiazepine antagonist but decreased the B_{\max} of a benzodiazepine agonist (Sweetnam and Tallman 1985). However, these changes were only about 20 percent. More notably, the augmentation of benzodiazepine binding to brain membranes by pentobarbital was abolished by deglycosylation (Sweetnam and Tallman 1985). In another study, glycopeptidase A, which cleaves the entire carbohydrate complex from the protein, and β -galactosidase, which removes only the terminal galactose residues, both increased the high affinity binding of [3 H]muscimol, apparently by eliminating the low-affinity sites (Kuriyama and Taguchi 1987). These studies suggest that the carbohydrate chains may be important for allosteric interactions between barbiturate and benzodiazepine receptors and for the interconversion of the low- and high-affinity states of the GABA receptor. Neither of these studies evaluated the role of glycosylation in the function of the chloride channel. It is possible that genetic differences in glycosylation could account for some of the genetic differences in GABA-activated chloride channels, but there are no data at present to support this speculation.

Genetic Studies of Actions of Ethanol on GABA-Activated Chloride Channels

General Background

The search for the neuropharmacologic basis of the actions of ethanol has been hampered by the lack of pharmacologic specificity of ethanol and the corresponding lack of pharmacologic tools to assist us in studying this drug. A particular problem is that when tested at large concentrations (200-600 mM), ethanol alters many neurochemical processes. Effects of concentrations below 100 mM are likely more relevant for intoxication and anesthesia, and a number of actions of such concentrations of ethanol have been identified. However, observation of a neurochemical change after *in vitro* exposure of brain tissue to reasonable concentrations of ethanol or after *in vivo* injection of nonlethal doses of ethanol does not ensure that the neurochemical change is actually important for the action of ethanol. A major challenge in alcohol research (and neuropharmacology in general) is to bridge the gap between neurochemistry and behavior so as to determine which neurochemical actions of a drug are responsible for behavioral changes.

Some ways to relate neurochemistry to behavior include correlation based on dose- (or concentration-) response relationships, potencies of different drugs or time courses for drug action. Each of these parameters is important, but at best they give evidence of correlation rather than causality. A more compelling approach is to relate genetic differences in drug response at the behavioral level to genetic differences in neurochemical responses. Alcohol researchers are fortunate to have available a number of rodent lines selected for differences in response to acute or chronic administration of ethanol. An example of this paradigm is our studies of effects of ethanol on GABA-activated chloride channels.

Genetic variation among rodent populations in response to ethanol has been successfully exploited by selective breeding programs. The goal of selective breeding is to generate divergent lines through the intermating of subjects that score similarly on a particular phenotype. The basic strategy is to start with a heterogeneous population and to breed individuals with high levels of

the desired phenotype to others with high levels, while individuals with low phenotypic levels are bred with other low-scoring individuals. Given a large heritable variance in the founding population, appropriate selection pressure, and enough selection generations, the procedure will produce two lines of subjects that differ markedly in the phenotype under selection (Falconer 1960). Ideally, the selection procedure should affect changes in the frequency of only those genes that are related to the expression of the selected phenotype (Crabbe and Belknap 1980). Therefore, differences between selected lines on some trait other than that being selected may indicate a relationship between the observed difference and the selection index.

Studies of LS and SS Mice

The LS and SS mice were selectively bred for differential sensitivity to the hypnotic effects of ethanol as measured by the duration of loss of the righting reflex (sleep time) (McClern and Kakihana 1973). By the 14th generation of selection, there was virtually no overlap in the sleep time distribution of the LS and SS lines. The difference between LS and SS mice in ethanol sleep time duration is primarily due to differences in neurosensitivity, since the lines exhibit a marked difference in ethanol-induced suppression of cerebellar Purkinje cell firing rate (Palmer 1985), whereas, ethanol elimination rates after intraperitoneal administration are virtually identical (Gilliam et al. 1985; Smolen et al. 1986).

Subsequent research has shown that these lines differ on a number of other response measures to both alcohol and other agents (see McIntyre and Alpern 1985 for a review). It has been suggested that differences between the lines in the GABA receptor-chloride ionophore system may be related to the selected differences in ethanol sleep time (Martz et al. 1983; McIntyre and Alpern 1985). We examined effects of muscimol (a GABA agonist) and ethanol on the uptake of $^{36}\text{Cl}^-$ by membrane vesicles prepared from cerebellum (Allan and Harris 1986) and cortex (Harris and Allan in press) of LS and SS mice. In both brain regions, muscimol was more potent in stimulating chloride flux in LS than in SS mice. Muscimol was also more potent in inhibiting the binding of $[\text{S}^{35}]\text{TBPS}$ in LS than SS mice. These neurochemical results are in agreement with the observation that GABA agonists are more potent in producing ataxia in LS than SS mice (Martz et al. 1983). However, these genetic differences in muscimol responses were

rather small and are difficult to relate to ethanol action. Of more interest is the observation that ethanol enhanced the effect of muscimol on chloride flux in LS but not in SS mice (Allan and Harris 1986). Thus, chloride channels of the SS mice appeared to be completely resistant to the action of ethanol. Pentobarbital also enhances muscimol-stimulated chloride flux, and it was of interest to compare its actions on LS and SS mice. Membranes from both lines of mice responded similarly to pentobarbital, consistent with behavioral studies showing that these lines are equally sensitive to the hypnotic effects of this drug. However, the LS mice are more sensitive than the SS mice to hypnotic effects of phenobarbital, and the chloride flux assay also demonstrated this line difference (unpublished data). Flunitrazepam also augments the muscimol-stimulated chloride flux, and the flunitrazepam concentration-response curve is shifted for LS as compared with SS membranes. For cortical membranes from LS mice, the 50 percent effective concentration (EC_{50}) for flunitrazepam is about 1 nM, whereas the EC_{50} for SS mice is about 15 nM (Allan and Harris in press). A similar difference for flunitrazepam sleep time was observed between the LS and SS lines (Wehner et al. this volume).

Thus, there was a rather striking correspondence between genetic differences in ethanol and barbiturate action in vivo and the effects of these agents on GABA-activated chloride channels in vitro for the LS and SS lines. These results suggest a relationship between ethanol hypnosis and the cerebellar GABA receptor-chloride channel. However, these concomitant responses may have been due either to pleiotropy or to a stochastic association among the genes that subserve each response. A significant amount of inbreeding has taken place in the LS and SS lines, which has contributed to differential gene fixation in the lines. One solution to this problem was to study other selected lines to determine whether the differences documented for LS and SS mice were peculiar to this selection or generalized to other species and selection criteria.

Studies of HAS and LAS Rats

The HAS and LAS rats were selected from an HS line of rats formed by an eight-way cross of inbred rat strains (Hansen and Spuhler 1984). The rats were in the eighth generation of selection at the time of our study, and almost

a threefold difference in ethanol sleep time and cerebellar Purkinje cell sensitivity to ethanol had been achieved (Palmer et al. 1987). Replicate lines in each direction of selection and unselected control lines have also been developed and maintained. The replicate lines provide another test of the validity of a genetic correlation and were also used in our study.

In contrast to the LS and SS lines, cortical membranes from both the HAS-1 and HAS-2 lines showed a response to muscimol similar to that of the LAS-1 and LAS-2 lines (Allan et al. 1988a). However, the differential response to ethanol seen in LS and SS lines was also seen in the HAS and LAS lines. Both the HAS-1 and HAS-2 lines showed an augmentation of muscimol-stimulated chloride flux by 10-30 mM ethanol, but neither the LAS-1 nor LAS-2 line showed any augmentation of flux by ethanol concentrations of 10-60 mM. This finding demonstrates that genetic differences in effects of muscimol on the channel (seen only in LS and SS lines) are not required for genetic differences in effects of ethanol on the channel (seen in LS/SS and HAS/LAS lines). These results also extend the studies with mice to another species (rats).

Studies of DS and DR Mice

The DS and DR mice developed by Gallaher et al. (1987) were selected for differences in the duration of diazepam-induced ataxia. Selection of these lines is still ongoing; in the ninth generation of the selection process, there was more than a fourfold difference between the lines in the duration of impairment by diazepam. These differences appear to be due to differences in CNS sensitivity and are not metabolically mediated. The DS mice, which are more behaviorally affected by diazepam, also have significantly lower brain diazepam concentrations at the time of recovery than do the less sensitive DR mice. The DS mice are also more sensitive to the ataxic actions of ethanol and other agents thought to act through the GABA receptor-chloride channel complex (Gallaher and Gionet 1988; Allan and Harris 1988b). Thus, our studies were designed to evaluate neurochemical differences in the GABAergic system between these mice that may account for the genetic differences in diazepam-, ethanol-, and phenobarbital-induced ataxia.

Although the selection procedure for DS/DR mice was quite different from that for LS/SS mice, the effects of drugs on chloride channels of DS/DR mice were quite similar to those observed with LS/SS mice. For example, the DS mice showed clear augmentation of GABA-stimulated chloride flux by ethanol or flunitrazepam, whereas the effects of these drugs were greatly attenuated in DR mice (Allan et al. 1988b). Likewise, the membranes from DS mice were somewhat more sensitive than those from DR mice to the effects of phenobarbital on chloride flux but displayed identical sensitivity to pentobarbital. These neurochemical results are consistent with genetic differences in behavioral sensitivity to these drugs.

Studies of HS Mice

Individual HS mice display marked differences in response to drugs, which, of course, is the basis for many of the selections discussed above. However, it is also possible to test individuals from HS stock for behavioral and neurochemical responses. This may be termed a "zero generation" selection.

We injected 100 HS mice with ethanol and measured their sleep times (Allan et al. 1988a). One week later, selected mice were killed and used as a source of cortical membranes for chloride flux assays. Membranes from the 10 mice that were behaviorally most sensitive to ethanol (long sleep times, HS-LS) displayed an augmentation of GABA-stimulated chloride flux by ethanol, but membranes from the 8 least sensitive mice (HS-SS) did not show this effect of ethanol. Thus, the response of HS-SS chloride channels to ethanol resembled that of the SS, LAS, and DR lines, indicating that the alcohol resistance phenotype seen in selected lines was present at the zero generation.

Conclusions from Genetic Studies

These studies of five different pairs of selected lines provide a remarkably consistent relationship between genetic insensitivity to ethanol at the behavioral and chloride channel level. Although not investigated as thoroughly, ethanol insensitivity appears to be linked to reduced sensitivity to flunitrazepam and phenobarbital but not to pentobarbital. In contrast, there is no consistent relationship between muscimol sensitivity and ethanol

sensitivity. These data suggest that the site of action of ethanol on the channel is closely related to the sites of action of benzodiazepines and phenobarbital but is distinct from the sites of action of pentobarbital and muscimol. An obvious question is whether binding properties of any of the receptors (GABA, benzodiazepine, or TBPS) associated with this channel complex differ among the selected lines in a manner consistent with genetic differences in effects of these agents on chloride channel function. Studies to date (Allan et al. 1988b; Allan and Harris 1986; Marley and Wehner 1987) have not demonstrated differences in receptor binding that could completely account for the genetic differences in chloride flux. For example, flunitrazepam is about 15 times more potent in enhancing GABA-stimulated chloride flux in DS than in DR membranes, yet the number and affinity of flunitrazepam binding sites is identical in membranes from the two lines (Harris and Allan in press). We speculate that the genetic differences reside in portions of the receptor-channel complex related to coupling ligand binding to channel function. We are currently attempting to define these differences by using molecular biologic approaches.

Despite the excellent qualitative relationship between effects of drugs on chloride flux and their effects on behavioral measures (hypnosis or ataxia), there are quantitative discrepancies between these measures. For example, SS mice are certainly affected by ethanol, and about twice the dose is required to give the same sleep time as is seen with LS mice. However, we were unable to obtain any augmentation of GABA-stimulated chloride flux by ethanol in membranes from SS mice. In addition, the LS/SS and DS/DR lines showed as large a differential behavioral response to phenobarbital and to ethanol, but the chloride flux demonstrated only a small genetic difference in phenobarbital compared with the large difference in ethanol action. More subtle discrepancies between behavior and neurochemistry can also be seen by comparing the HS-LS/HS-SS and LS/SS lines. The difference between the HS-LS/HS-SS mice in ethanol augmentation of muscimol-stimulated $^{36}\text{Cl}^-$ flux is equivalent to the difference observed between the LS and SS mice, although the genetic difference in ethanol sleep time is much greater for the LS and SS mice. For example, at a 3.5-g/kg dose of ethanol, LS mice sleep approximately 130 min, whereas SS mice do not lose the righting reflex at this dose. In our studies, this same dose of ethanol produced average sleep times of 123 min in HS-LS mice and 38 min in HS-SS mice. Thus, although the founding population (HS) for the two lines (LS and SS)

already differed maximally in sensitivity of the GABA-operated chloride channel to ethanol, the sleep time differences in the extremes of the HS distribution are not as great as the differences in LS and SS lines. This suggests that further selection of the LS and SS lines produced changes in the frequencies of genes controlling ethanol sleep time that are separate from those genes directly involved in the sensitivity of the GABA-operated chloride channel to ethanol. Involvement of additional genes in ethanol narcosis is also suggested by the differences in the concentration-response relationship for augmentation of chloride flux *in vitro* and production of anesthesia *in vivo*. Maximal enhancement of chloride flux occurred with 10-20 mM ethanol, whereas concentrations necessary for anesthesia (50-100 mM) produced little or no enhancement of chloride flux. This finding may indicate that actions of ethanol on the chloride channel are more important for low-dose effects of ethanol (e.g., locomotor stimulation) than for anesthesia. In this regard, it is clear that animals selected for genetic differences in ethanol anesthesia also differ in effects of ethanol on other behaviors (see Collins 1981 for review). Another possibility is that our *in vitro* assay conditions do not accurately measure the function of the chloride channel *in vivo*. Activation and inactivation of the chloride flux occurs on a millisecond time scale (Cash and Subbarao 1987), and the ethanol concentration-response relationship obtained at these times may not be the same as that measured with our 3-s flux time.

Overall Conclusions

These results indicate that the GABA-activated chloride channel is an important site of action of ethanol (and benzodiazepines). A key point is that not all GABA-activated chloride channels are equally sensitive to these drugs. This is clearly seen in lines of animals selected for sensitivity to ethanol or benzodiazepines in which there are genetic differences in channel sensitivity. There is also evidence that channels in different brain regions, and perhaps different neurons within a region, are not composed of the same subunits and may not be affected equally by ethanol or benzodiazepines. There are several possible mechanisms for differential drug sensitivity of GABA-activated chloride channels: (1) a modulator (e.g., calcium or cAMP) could convert the complex to a drug-sensitive or -insensitive form, (2) sequence differences of the multiple subunits of the channel could determine

drug sensitivity, or (3) different carbohydrate residues (glycosylation) could alter the drug sensitivity of the channel.

Recent development of biochemical and molecular approaches to study of chloride channel structure and function, including cloning of genes coding for the channel subunits, should allow alcohol researchers to distinguish between these three possibilities. One can hope that this sort of information will allow us to understand the actions of ethanol at a truly molecular level.

Summary

We review the effects of drugs on the γ -aminobutyric acid (GABA)-activated chloride channels of brain and suggest this complex as a site of action for ethanol and as a locus for genetic diversity in ethanol sensitivity. Several groups have reported that ethanol augments the GABA-stimulated chloride flux of isolated brain membranes assayed in vitro. We asked whether genetic differences in acute sensitivity to ethanol (and related drugs such as benzodiazepines and barbiturates) are due to differences in the functioning of the GABA-activated chloride channel. We tested animals from five different pairs of selected lines and found in all cases that a reduced behavioral sensitivity to ethanol was accompanied by a lack of effect of ethanol on chloride flux. Reduced sensitivity to ethanol action also generalized to reduced effects of flunitrazepam on chloride flux. Genetic differences in ethanol sensitivity did not result in differences in pentobarbital action in vivo or in vitro. These studies suggest that the GABA-activated chloride channel is one, but not the only, neurochemical process responsible for genetic differences in acute sensitivity to ethanol. Possible mechanisms for the effects of ethanol on chloride channels and genetic differences in susceptibility to ethanol are discussed.

References

- Allan, A.M.; Gallaher, E.J.; Gionet, S.E.; and Harris, R.A. Genetic selection for benzodiazepine ataxia produces functional changes in the gamma-aminobutyric acid receptor chloride channel complex. *Brain Res* 452:118-126, 1988a.

- Allan, A.M.; and Harris, R.A. Gamma-aminobutyric acid and alcohol actions: Neurochemical studies of long sleep and short sleep mice. *Life Sci* 39:2005-2015, 1986.
- Allan, A.M., and Harris, R.A. Acute and chronic ethanol treatments alter GABA-receptor operated chloride channels. *Pharmacol Biochem Behav* 27:665-670, 1987a.
- Allan, A.M., and Harris, R.A. Involvement of neuron chloride channels in ethanol intoxication, tolerance and dependence. In: M. Galanter, ed. *Recent Developments in Alcoholism*. Vol. V. New York: Plenum Press, 1987b. pp. 313-325.
- Allan, A.M.; Spuhler, K.P.; and Harris, R.A. Gamma-aminobutyric acid-activated chloride channels: Relationship to genetic differences in ethanol sensitivity. *J Pharmacol Exp Ther* 244:866-870, 1988b.
- Bureau, M., and Olsen, R.W. Gamma-aminobutyric acid/benzodiazepine receptor protein carries binding sites for both ligands on both two major peptide subunits. *Biochem Biophys Res Commun* 153:1006-1011, 1988.
- Cash, D.J., and Subbarao, K. Channel opening of gamma-aminobutyric acid receptor from rat brain: Molecular mechanisms of the receptor responses. *Biochemistry* 26:7562-7570, 1987.
- Celentano, J.J.; Gibbs, T.T.; and Farb, D.H. Ethanol potentiates GABA- and glycine-induced chloride currents in chick spinal cord neurons. *Brain Res* 455:377-380, 1988.
- Collins, A.C. A review of research using the short-sleep and long-sleep mice. In: McClearn, G.E.; Dietrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. ADM (81-1113). Washington, DC: Supt. of Docs. U.S. Govt. Print. Off., 1981. pp. 161-170.
- Crabbe, J.C., and Belknap, J.K. Pharmacogenetic tools in the study of drug tolerance and dependence. *Subst Alcohol Actions Misuse* 1:385-413, 1980.

- Davidoff, R.A. Alcohol and GABA presynaptic inhibition in an isolated spinal cord preparation. *Arch Neurol* 28:60-63, 1973.
- Dudek, B.C., and Abbott, M.E. A biometrical genetic analysis of ethanol response in selectively bred long-sleep and short-sleep mice. *Behav Genet* 14:1-19, 1984.
- Falconer, D.S. *Introduction to Quantitative Genetics*. New York: Roland Press, 1960.
- Frye, G.D., and Breese, G.R. GABAergic modulation of ethanol-induced motor impairment. *J Pharmacol Exp Ther* 223:750-756, 1982.
- Gage, P.W., and Robertson, B. Prolongation of inhibitory postsynaptic currents by pentobarbitone, halothane and ketamine in CA1 pyramidal cells in rat hippocampus. *Br J Pharmacol* 85:675-681, 1985.
- Gallaher, E.J., and Gionet, S.E. Initial sensitivity and tolerance to ethanol in mice genetically selected for diazepam sensitivity. *Alcohol Clin Exp Res* 12:77-80, 1988.
- Gallaher, E.J.; Hollister, L.E.; Gionet, S.E.; and Crabbe, J.C. Mouse lines selected for genetic differences in diazepam sensitivity. *Psychopharmacology* 93:25-30, 1987.
- Gilliam, D.M.; Phillips, T.J.; and Dudek, B.C. A comparison of ethanol absorption and narcosis in long- and short-sleep mice following intraperitoneal or intragastric ethanol administration. *Alcohol* 2:655-658, 1985.
- Hansen, C., and Spuhler, K. Development of a NIH genetically heterogeneous rat stock. Symposium: The development of a rat model for pharmacogenetic research in alcoholism. *Alcoholism Clin Exp Res* 8:477-479, 1984.
- Harris, R.A., and Allan, A.M. Genetic differences in coupling of benzodiazepine receptors to chloride channels. *Brain Res* 490:26-32, 1989.

- Harris, R.A.; Allan, A.M.; Daniell, L.C.; and Nixon, C. Antagonism of ethanol and pentobarbital actions by benzodiazepine inverse agonists: Neurochemical studies. *J Pharmacol Exp Ther* 247:1012-1017, 1988.
- Harrison, N.L., and Lambert, N.A. The effects of protein kinase activators on GABA_A receptor function in cultured rat hippocampal neurons. [Abstract in the Proceedings of the Physiological Society-Cardiff]. *J Physiol* Vol. 412, 17 P, 1989.
- Harrison, N.L.; Majewska, M.D.; Harrington, J.W.; and Barker, J.L. Structure-activity relationships for steroid interaction with the gamma-aminobutyric acid A receptor complex. *J Pharmacol Exp Ther* 241:346-353, 1987.
- Houmed, K.M.; Bilbe, G.; Smart, T.G.; Constanti, A.; Brown, D.A.; Barnard, E.A.; and Richards, B.M. Expression of functional GABA, glycine and glutamate receptors in *Xenopus* oocytes injected with rat brain mRNA. *Nature* (London) 310:318-321, 1984.
- Huganir, R.L.; Delcour, A.H.; Greengard, P.; and Hess, G.P. Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. *Nature* (London) 321:774-776, 1988.
- Huidobro-Toro, J.P.; Bleck, V.; Allan, A.M.; and Harris, R.A. Neurochemical actions of anesthetic drugs on the gamma-aminobutyric acid receptor-chloride channel complex. *J Pharmacol Exp Ther* 242:963-969, 1987.
- Inoue, M.; Oomura, Y.; Yakushiji, T.; and Akaike, N. Intracellular calcium ions decrease the affinity of the GABA receptor. *Nature* (London) 324:156-158, 1986.
- Kuriyama, K., and Taguchi, J. Glycoprotein as a constituent of purified gamma-aminobutyric acid/benzodiazepine receptor complex: Structures and physiological roles of its carbohydrate chain. *J Neurochem* 48:1897-1903, 1987.

- Levitan, E.S.; Schofield, P.R.; Burt, D.R.; Rhee, L.M.; Wisden, W.; Kohler, M.; Fujita, N.; Rodriguez, H.R.; Stephenson, A.; Darlison, M.G.; Barnard, E.A.; and Seeburg, P.H. Structural and functional basis for GABA_A receptor heterogeneity. *Nature* (London) 335:76-79, 1988.
- Lister, R.G., and Nutt, D.J. Is Ro 15-4513 a specific alcohol antagonist? *Trends Neurosci* 10:223-225, 1987.
- Mamalaki, C.; Stephenson, F.A.; and Barnard, E.A. The GABA_A/benzodiazepine receptor is a heterotetramer of homologous α and β subunits. *EMBO J* 6:561-565, 1987.
- Mancillas, J.R.; Siggins, G.R.; and Bloom, F.E. Systemic ethanol: Selective enhancement of responses to acetylcholine and somatostatin in hippocampus. *Science* 231:161-163, 1986.
- Marley, R.J., and Wehner, J.M. GABA enhancement of flunitrazepam binding in mice selectively bred for differential sensitivity to ethanol. *Alcohol Drug Res* 7:25-40, 1987.
- Martz, A.; Dietrich, R.A.; and Harris, R.A. Behavioral evidence for the involvement of gamma-aminobutyric acid in the actions of ethanol. *Eur J Pharmacol* 89:53-62, 1983.
- McClearn, G.E., and Kাকihana, R. Selective breeding for ethanol sensitivity in mice. *Behav Genet* 3:409-410, 1973.
- McIntyre, T.D., and Alpern, H.P. Reinterpretation of the literature indicates differential sensitivities of long-sleep and short-sleep mice are not specific to alcohol. *Psychopharmacology* 87:379-389, 1985.
- Mereu, G., and Gessa, G.L. Low doses of ethanol inhibit the firing of neurons in the substantia nigra, pars reticulata: A GABAergic effect? *Brain Res* 360:325-330, 1985.
- Morrow, A.L.; Suzdak, P.D.; Karanian, J.W.; and Paul S.M. Chronic ethanol administration alters gamma-aminobutyric acid, pentobarbital and

- ethanol-mediated $^{36}\text{Cl}^-$ uptake in cerebral cortical synaptoneurosomes. *J Pharmacol Exp Ther* 246:158-164, 1988.
- Nestoros, J.N. Ethanol specifically potentiates GABA-mediated neurotransmission in feline cerebral cortex. *Science* 209:708-710, 1980.
- Nishio, M., and Narahashi, T. Ethanol enhances a component of GABA-gated chloride channel currents in rat dorsal root ganglion neurons. *Soc Neurosci Abst* 14:642, 1988.
- Palmer, M.R. Neurophysiological mechanisms in the genetics of ethanol sensitivity. *Soc Biol* 32:241-254, 1985.
- Palmer, M.R.; Wang, Y.; Fossum, L.H.; and Spuhler, K. Genetic correlation of ethanol-induced ataxia and cerebellar Purkinje neuron depression in inbred rat strains and selected lines of rats. *Alcoholism Clin Exp Res* 11:494-501, 1987.
- Pritchett, D.; Schofield, P.R.; Sontheimer, H.; Ymer, S.; Kettermann, H.; and Seeburg, P. GABA receptor cDNAs expressed in transfected cells and studied by patch-clamp and binding assay. *Soc Neurosci Abst* 14:641, 1988.
- Schofield, P.R.; Darlison, M.G.; Fujita, N.; Burt, D.R.; Stephenson, F.A.; Rodriguez, H.; Rhee, L.M.; Ramachandran, J.; Reale, V.; Glencorse, T.A.; Seeburg, P.H.; and Barnard, E. Sequence and functional expression of the GABA_A receptor shows a ligand-gated receptor super-family. *Nature* (London), 238:221-227, 1987.
- Sigel, E., and Baur, R. Allosteric modulation by benzodiazepine receptor ligands of the GABA_A receptor channel expressed in *Xenopus* oocytes. *J Neurosci* 8:289-295, 1988a.
- Sigel, E., and Baur, R. Activation of protein kinase C differentially modulates neuronal Na⁺, Ca²⁺, and gamma-aminobutyrate type A channels. *Proc Natl Acad Sci USA* 85:6192-6196, 1988b.

- Smolen, A.; Marks, M.J.; Smolen, T.N.; and Collins, A.C. Dose and route of administration alter the relative elimination of ethanol by long-sleep and short-sleep mice. *Alcoholism Clin Exp Res* 10:198-204, 1986.
- Stelzer, A.; Kay, A.R.; and Wong, R.K.S. GABA_A-receptor function in hippocampal cells is maintained by phosphorylation factors. *Science* 241:339-341, 1988.
- Supavilai, P.; Cortes, R.; Palacios, J.M.; and Karobath, M. Evidence for the existence of a GABA_A/chloride ion channel complex in mammalian brain which does not contain benzodiazepine receptor sites. In: Biggio, G.; Spano, P.F.; Toffano, G.; and Gessa, G.G., eds. *Modulation of Central and Peripheral Transmitter Function*. Fidia Research Series, Symposia in Neuroscience III. Padova: Liviana Press, 1986.
- Suzdak, P.D.; Glowa, J.R.; Crawley, J.N.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. A selective imidazobenzodiazepine antagonist of ethanol in the rat. *Science* 234:1243-1247, 1986a.
- Suzdak, P.D.; Schwartz, R.D.; and Paul, S.M. Ethanol stimulates gamma-aminobutyric acid receptor mediated chloride transport in rat brain synaptoneurosomes. *Proc Natl Acad Sci USA* 83:4071-4075, 1986b.
- Sweetnam, P.M., and Tallman, J.F. Regional difference in brain benzodiazepine receptor carbohydrates. *Mol Pharmacol* 29:299-306, 1985.
- Taleb, O.; Trouslard, J.; Demeneix, B.A.; Feltz, P.; Bossu, J.-L.; Dupont, J.-L.; and Feltz, A. Spontaneous and GABA-evoked chloride channels on pituitary intermediate lobe cells and their internal Ca requirements. *Pfluegers Arch* 409:620-631, 1987.
- Ticku, M.K., and Kulkarni, S.K. Molecular interactions of ethanol with GABAergic system and potential of Ro 15-4513 as an ethanol antagonist. *Pharmacol Biochem Behav* 30:501-510, 1988.

Discussion

OLSEN: If I could comment on the flunitrazepam activation of the muscimol-dependent increase in Cl flux. In the LS mice, the ED_{50} for flunitrazepam was in the nanomolar range and a receptor occupancy of 20 percent. This is very unusual, and so it is not so much the SS that are different, but it is the LS that are unusual.

HARRIS: This is the first time that someone has estimated spare receptors using chloride flux. In in vivo studies (*Nature* [London] 281:688, 1979; *J Pharmacol Exp Ther* 240:516, 1987) where the in vivo binding was measured and a behavioral task was used, they found that only about one-third of the receptors had to be occupied to get the response.

Behavioral Studies of GABAergic Responses in LS and SS Mice: Are Ethanol Sensitivity and Responses to GABAergic Agents Regulated by Common Mechanisms?¹

*Jeanne M. Wehner,^{2,3} Barbara J. Martin,^{2,4}
R. J. Marley,^{2,3,5} and June I. Pounder²*

Introduction

Numerous behavioral and biochemical studies implicate the γ -aminobutyric acid (GABA)ergic system in the mediation of some of the actions of ethanol. GABA mimetics prolong the duration of the loss of ethanol-induced anesthesia (Liljequist and Engle 1982) and increase motor incoordination produced by ethanol (Frye and Breese 1982; Martz et al. 1983), and there

¹This study was supported by grant AA03527 from the National Institute on Alcohol Abuse and Alcoholism.

²Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309.

³School of Pharmacy, University of Colorado, Boulder, CO 80309.

⁴Department of Psychology, University of Colorado, Boulder, CO 80309.

⁵Present Address: Department of Psychiatry, Yale University, New Haven, CN 06520.

appears to be some cross-tolerance between ethanol and benzodiazepines (Le et al. 1986). Biochemical studies have indicated that ethanol potentiates GABA-stimulated Cl^- flux (Suzdak et al. 1986; Ticku et al. 1986) and enhances benzodiazepine binding both in vitro and in vivo (Burch and Ticku 1980; Miller et al. 1988).

We and others have used a pharmacogenetic approach to study the relationship between ethanol and GABAergic function. Previous studies have shown that the long sleep (LS) and short-sleep (SS) mice, lines of mice selected for their differential responses to a hypnotic dose of ethanol (McClearn and Kakihana 1981), differ in ethanol potentiation of muscimol-stimulated Cl^- flux (Allan and Harris 1986). Furthermore, Allan et al. (1988b) showed a correlation between the degree of this potentiation and length of sleep time response in the heterogenous stock (HS) of mice from which the LS and SS mice were originally selected. Finally, the lines of mice resulting from selection for sensitivity to the ataxic properties of diazepam, the diazepam-resistant (DR) and diazepam-sensitive (DS) mice, differ in their behavioral responses to ethanol (Gallagher and Gionet 1988) as well as ethanol potentiation of muscimol-stimulated Cl^- flux (Allan et al. 1988b). These lines of evidence suggest that common genes may regulate sensitivity to ethanol and GABAergic agents. However, it is unclear whether this commonality holds for all GABAergic responses. Moreover, because of the known regional heterogeneity of the benzodiazepine receptor, it is not known how this variation is related to regional control of GABAergic and ethanol responses.

Our strategy has been to screen LS and SS mice on a number of behaviors regulated by the GABAergic system, define receptor characteristics in these mice, and then ultimately to correlate receptor characteristics with behavioral responses. However, because of the nature of the selection process, differences that might exist in neurotransmitter systems between the two lines cannot be used as de facto proof of shared genes. Fortuitous linkages resulting from random genetic drift may occur between ethanol responsiveness and specific characteristics of the GABAergic system. For this reason, potential differences must be pursued further by using experimental strategies designed for genetic correlations. These include the use of inbred strains, heterogenous stocks of mice, or recombinant inbred

strains (RIs). For our studies, we are using the LSxSS RIs that were recently developed at the Institute for Behavioral Genetics at the University of Colorado Alcohol Research Center (DeFries et al. 1989).

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) and therefore mediates diverse responses. For our assessment of the relationship between GABAergic function and ethanol sensitivity, we have used seizure susceptibility to a chemical induction of seizure activity via (1) inhibition of glutamate decarboxylase (GAD) activity by 3-mercaptopropionic acid (3MP) and (2) binding of the GABA antagonist bicuculline to the receptor. Latencies to stages of seizure activity were measured in LS and SS mice and LSxSS RIs.

Benzodiazepines produce many behavioral effects, including muscle relaxation and sedation, as well as anticonvulsant and anxiolytic actions (Haefely et al. 1983). To explore possible associations between benzodiazepine action and ethanol sensitivity, we examined the sedative, anticonvulsant, and anxiolytic properties of benzodiazepines. Sedative actions were determined by measuring the duration of the loss of the righting response (sleep time) after high doses of flurazepam. Anticonvulsant effects were analyzed by measuring the increased latency to seizures after an intraperitoneal (IP) injection of either flurazepam or diazepam.

Measurement of anxiolytic actions of benzodiazepine and ethanol has been difficult. It has been proposed that exploratory tests in a novel environment provide the best way to measure these actions in the mouse (File 1985), but interpretation of such tests is confounded by the fact that ethanol and other anxiolytics may produce locomotor activation (Sanders 1976; Dudek et al. 1984). For this reason, we have used the elevated plus-maze in which behavioral activation can be distinguished from anxiolytic actions of drugs (Pellow et al. 1985).

Methods

Animals

Male and female LS and SS mice and LSxSS RIs were obtained from

the Institute for Behavioral Genetics, University of Colorado. All mice were weaned at 25 days of age and housed with two to five like-sex littermates. Animals were maintained on a 12-hr light/dark cycle (lights on from 7 a.m. until 7 p.m.) and were permitted free access to food (Wayne Lab Blox) and water. All testing was performed between 55 and 90 days of age. Animals were placed in the testing room for 2-12 hr before behavioral testing.

Measurement of Sleep Time

The sedative-hypnotic effect of flurazepam was measured by using the same procedures that were employed during the selection of the LS and SS mice as outlined in detail previously (Marley et al. 1986). Dose-response curves for flurazepam ranging from 75 to 225 mg/kg were generated. Flurazepam was prepared fresh daily in saline. All experiments were conducted in a temperature-controlled room maintained at 26 °C. Observation periods were limited to 3 hr. Dose-response curves were analyzed by least-square linear regression techniques to determine slope and ED_{60} . The ED_{60} value represents the dose predicted to produce a 60-min sleep time. Comparisons of ED_{60} s were made by using Student's T-test.

Body Temperature Analysis

Body temperature was measured as previously described (Howerton et al. 1983). Flurazepam-induced decreases in body temperature were determined by using analysis of variance (ANOVA) techniques to evaluate effect of dose and population.

Seizure Testing

Seizure susceptibility was determined by using two agents: 3MP (15-55 mg/kg) and bicuculline (1-6 mg/kg). Dose-response curves for each agent were generated for latencies to either onset of seizures, clonus, or tonus as previously described (Marley and Wehner 1987a; Freund et al. 1987). Genetic comparisons for susceptibility were made by using ANOVA. For the bicuculline-induced seizures in the RIs, the latencies to clonus and tonus at 4 mg/kg provided a distinct separation among the 22 strains tested. Therefore, these data points were used to generate strain frequency histograms.

Determination of the Anticonvulsant Properties of Flurazepam and Diazepam

The anticonvulsant effects of benzodiazepines were assessed for 3MP-induced seizures (15-55 mg/kg) or at a dose of 3MP (55 mg/kg) that equalized the latency to seizure onset between LS and SS mice (Marley and Wehner 1987a). The mice were injected with flurazepam (1-6 mg/kg) or saline 30 min before the administration of 3MP. Flurazepam was dissolved in saline, whereas diazepam was dissolved in acidified water (pH 2.9). For diazepam, acidified water served as the control vehicle. Latencies to seizure onset were determined as described previously (Marley and Wehner 1987a) and were analyzed by ANOVA.

Determination of Anxiolytic Properties of Ethanol and Diazepam

The elevated plus-maze apparatus was used as characterized by Lister (1988). Animals were injected IP with either ethanol (20 percent [wt/vol]), diazepam (dissolved in acidified saline), or appropriate vehicle (0.01 ml/g). After 20 min, the animals were tested in the elevated plus-maze as previously described (Stinchcomb et al. in press). The percentage of time in the closed versus open arms, the total number of crosses into all arms, and the crosses into closed versus open arms were scored by using a videotape of a 5-min test session. Data were analyzed by ANOVA techniques.

GABA Enhancement of ³H-flunitrazepam Binding

Cortical and cerebellar tissue were assayed for GABA enhancement as described previously (Marley and Wehner 1987a).

Corticosterone Levels

Plasma corticosterone levels were determined as described previously (Martin and Wehner 1988).

Results and Discussion

Sedative-Hypnotic Effects

LS mice were more sensitive than SS mice to high doses of flurazepam (figure 1), as demonstrated by the dose-response curves that do not overlap and the comparison of ED_{60} values (the predicted dose to produce a 60-min sleep time). The ED_{60} dose in LS mice was 143.9 ± 16.5 mg/kg, as compared with 321.2 ± 15.1 mg/kg in SS mice ($t_{81} = 5.1$, $P < 0.001$). There were no differences between the slopes of the dose-response curves in the two lines of mice.

This differential response between LS and SS mice to flurazepam has also been observed with other benzodiazepines (McIntyre and Alpern 1986) and is similar to that observed for ethanol; i.e., LS mice are of the more sensitive genotype. This result supports the hypothesis that common genes regulate the sedative responses to ethanol and benzodiazepines in these selected lines of mice. If this is the case, a common biochemical mechanism might be expected to mediate these CNS responses. Allan and Harris (1986) have observed that ethanol potentiates muscimol-stimulated Cl^- flux in cerebellar microsacs from LS but not SS mice. Furthermore, the relationship between this biochemical measure and the behavioral responses to the sedative properties of ethanol and benzodiazepines is supported by their investigations (Allan et al. 1988b) showing that ethanol potentiation of Cl^- flux is greater in the longer sleeping members of an HS population of mice, in the high-alcohol-sensitive (HAS) selected rats, and in another selected line, DS mice (Allan et al. 1988); Harris and Allan 1989). This differential sensitivity also extends to benzodiazepines, since a dose-response analysis of flunitrazepam potentiation of Cl^- flux indicated that LS cerebellar microsacs were more sensitive (Harris and Allan 1989). It therefore appears that this cerebellar biochemical marker provides a likely mechanism for the mediation of the sleep time response.

Using electrophysiologic analysis, Hoffer and colleagues have shown that the cerebellum is of regional significance in mediating the hypnotic response to

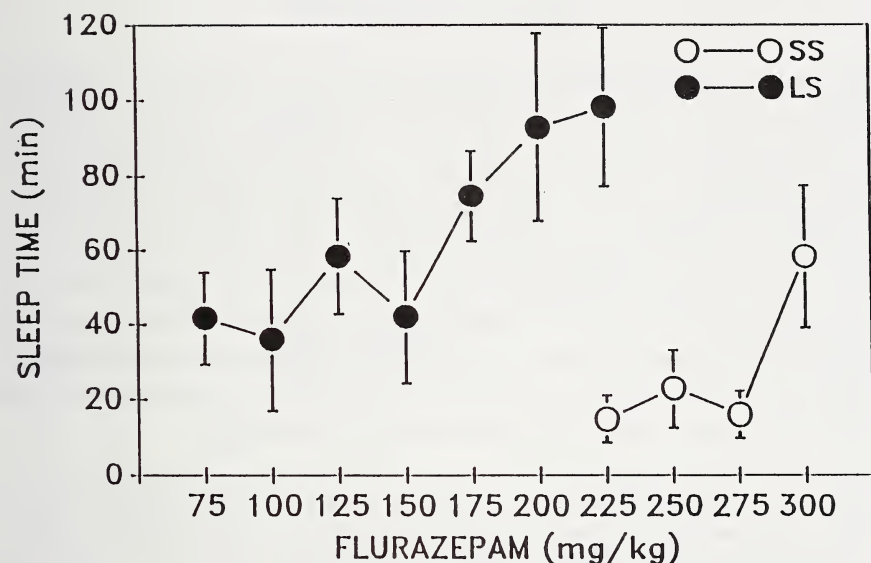


Figure 1.—Flurazepam-induced loss of righting response in female LS and SS mice. Each point represents the mean \pm standard error of the mean (SEM) duration of loss of righting response for seven to nine mice.

ethanol (Sorenson et al. 1980). None of these studies, however, have shown the molecular nature of the benzodiazepine receptor involved in this coupled response in the cerebellum. Although the comparison between LS and SS cerebellar benzodiazepine receptors awaits the completion of the cloning studies of Sikela and co-workers (this volume), this comparison is of potential interest since LS and SS cerebellar benzodiazepine receptors differ in GABA protection of heat inactivation of flunitrazepam binding, a potential indicator of molecular differences in either the membrane or the receptor protein itself (Marley et al. 1988). The evidence relating GABAergic mediation of sleep time response to ethanol sensitivity strongly supports the hypothesis that a genetic and mechanistic commonality exists between regulation of GABAergic function in the cerebellum and the sedative-hypnotic responses to ethanol and benzodiazepines. What remains to be determined from behavioral studies using the RIs is whether this relationship between benzodiazepine and ethanol sensitivity will be maintained over a broader range of genetically diverse populations of mice.

Hypothermic Responses

In mice, an IP injection of ethanol produces a hypothermic response (Howerton et al. 1983). LS mice are more sensitive than SS mice. Although different brain regions may regulate the hypothermic response and the sedative-hypnotic response, the degree of membrane perturbation may be regulated by membrane characteristics that are common to these two behaviors. It has been proposed that differential sleep time between LS and SS mice is dependent on the lipid solubility of the agent used. An examination of this hypothesis demonstrated a significant positive correlation between the partition coefficient of the sedative hypnotics and the differential sleep times in LS and SS mice (Marley et al. 1986). This hypothesis has not been tested rigorously by using other behavioral measures.

Injection of benzodiazepines also produce long-lasting hypothermia in mice. We examined this response as another indicator of the sensitivity of the GABAergic system and to determine ultimately whether the hypothesis concerning differential effects of drugs of varying solubilities was supported by a physiologic response that appears to be correlated to sleep time. We initially used flurazepam, a water-soluble benzodiazepine, since it would be expected to produce the most pronounced difference between LS and SS mice based on the lipid solubility hypothesis. A dose-response curve for the induction of hypothermia is shown in figure 2. In contrast to the other behaviors examined, there was no difference in response between LS and SS mice.

It appears from these limited data that common genes do not regulate GABAergic responses to benzodiazepine-induced and ethanol-induced hypothermia. Furthermore, it appears on the basis of these data and a more extensive survey of alcohols and benzodiazepines (A.C. Collins, J.M. Wehner, and R.J. Marley, unpublished data) that the hypothetical differential response in LS and SS mice based on lipid solubilities will not extrapolate to hypothermia.

Seizure Susceptibility

Phillips and Dudek (1983) observed that SS mice were more sensitive than

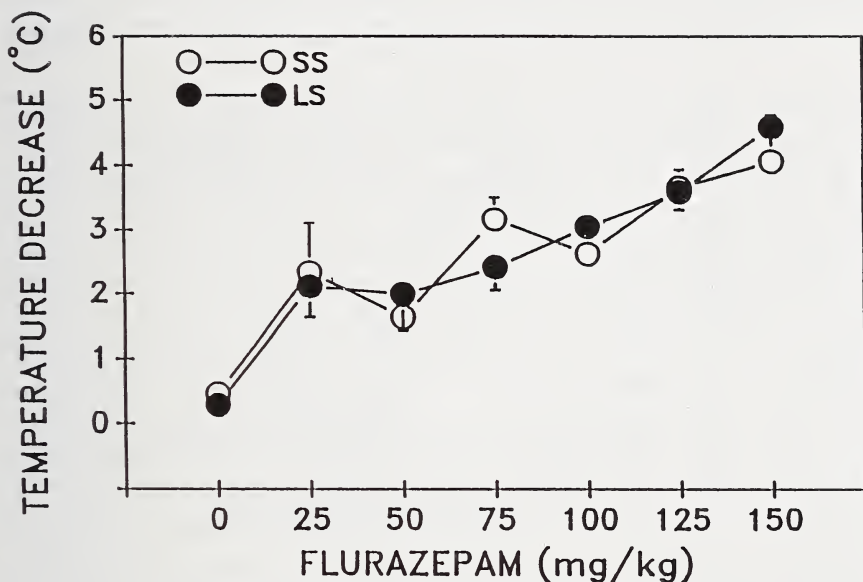


Figure 2.—Flurazepam-induced hypothermia in female LS and SS mice. Each points reflects the mean \pm SEM decrease in body temperature 30 min after the administration of flurazepam for six mice.

LS mice to bicuculline-induced seizures, using stocks of these selected lines maintained for several years at the University of New York at Albany. In their study, bicuculline-induced myoclonus and clonus occurred after a shorter latency in SS mice than in LS mice. Because this pattern fits the LS/SS sensitivity pattern exhibited by the sleep time response, we also examined seizure susceptibility to bicuculline and have reproduced their results in stocks of LS and SS mice maintained at the University of Colorado (figure 3).

If the genes regulating GABAergic mechanisms mediating seizure susceptibility are common to those regulating initial sensitivity to ethanol, then a correlational analysis of LSxSS RIs should clarify this relationship. Of the 27 RIs available at the Colorado Alcohol Research Center, 22 were tested for bicuculline seizure susceptibility over a dose range of 2-5 mg/kg (data not shown). There was a significant genetic variation in the response of

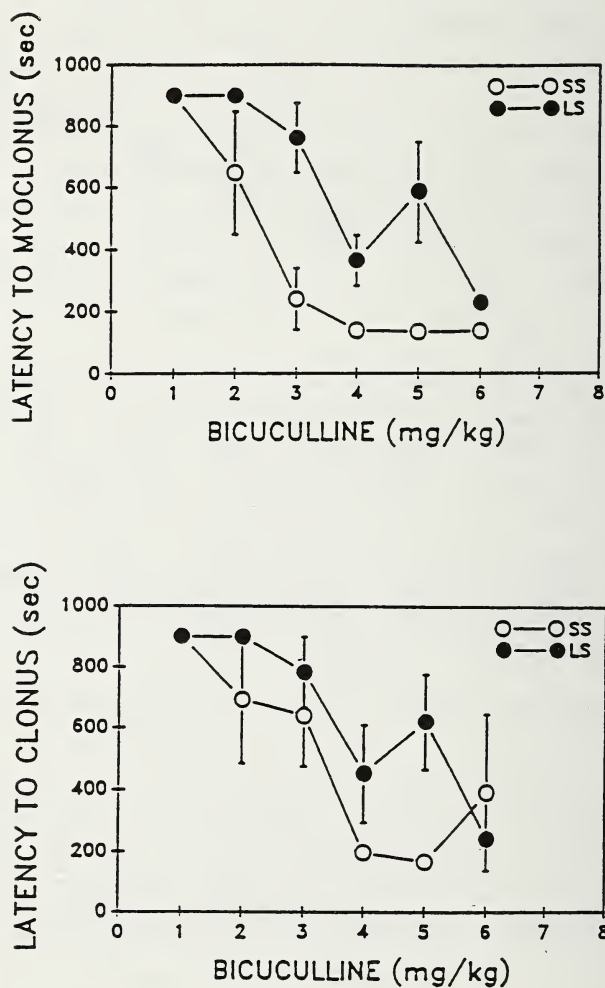


Figure 3.—Latency to the onset of seizures induced by bicuculline in female LS and SS mice. Each point represents the mean \pm SEM of three to four mice.

these 22 RIs over this dose range [$F_{(21,1225)}=7.8, P<0.001$], and females had a significantly lower seizure threshold than males [$F_{(1,1225)}=83.2, P<0.001$].

To perform the correlational analysis, a single measurement was needed to describe the differential sensitivity. The latency to either clonus or tonus at 4 mg/kg exhibited a distinct separation among the populations tested and was used for further analysis. The frequency histogram that describes this separation among strains is shown in figure 4. For these studies, only clonus and tonus were measured; in our laboratory, these seizure stages have proven to be more reliable measures of seizure susceptibility than myoclonus. The frequency histogram clearly indicates that there is variation in seizure susceptibility, but that more strains are sensitive than are resistant. Furthermore, there is no simple bimodal distribution among the strains that would indicate a single gene phenomenon. In addition, LS and SS latencies fall within the extremes of the histogram. This visual observation was supported by calculating "effective factors" by the equation $R^2=4(1/2V_A)$ (DeFries et al. submitted). For clonus, three to four "effective factors" were calculated, supporting the conclusion that more than one gene controls this behavior. This calculation could not be performed for tonus data because some strains had mean values at a ceiling of 900 s with no variance.

The correlation between sleep time scores and latency to clonus or tonus is shown in figure 5. No significant correlations were observed for either stage of seizures in males or females. These results indicate that common genes do not regulate sensitivity to the sedative-hypnotic effects of ethanol and seizure susceptibility to bicuculline. Although the bicuculline sensitivity of LS and SS mice appeared to conform to the pattern predicted by ethanol sensitivity, it is clear from these correlational analyses that this measure is a "false-positive" as described by Deitrich (this volume).

Because bicuculline is a GABA antagonist and binds at the GABA site of the receptor complex, it might be expected that this type of seizure production would be controlled by a single-gene mechanism. The results generated by using the RIs do not support this hypothesis. The neural substrates regulating behavioral manifestations of seizures must involve more complex interactions subsequent to antagonist binding, including pathways regulating

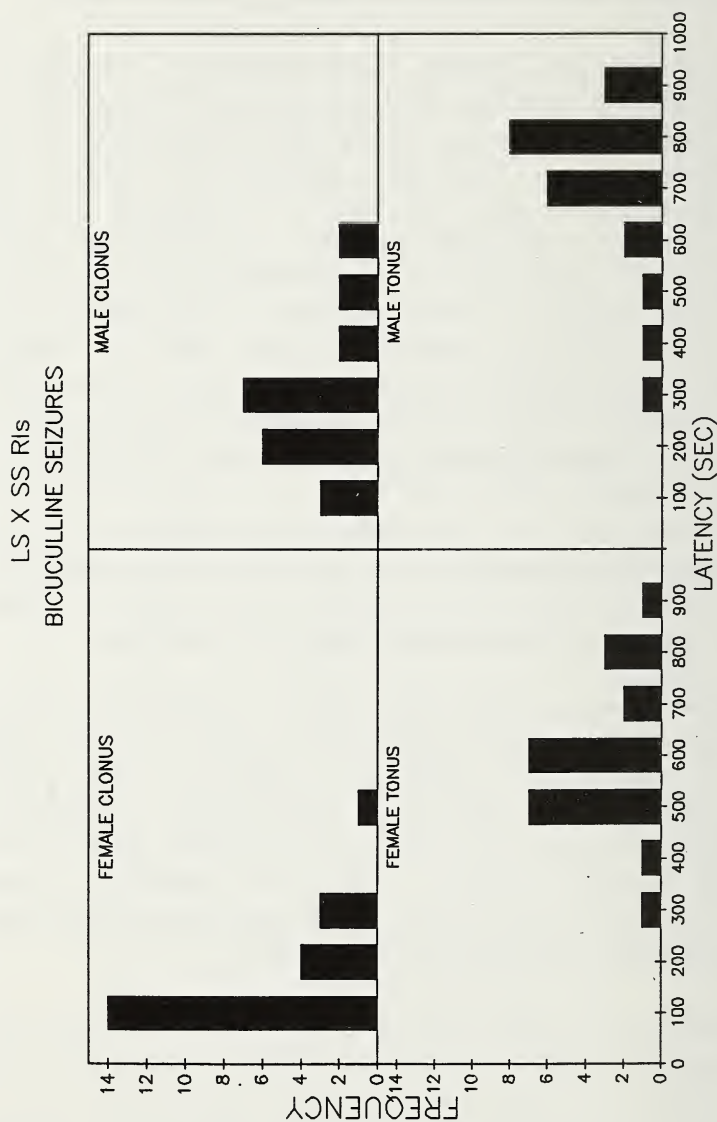


Figure 4.—Strain frequency histogram of clonic and tonic seizures produced by 4.0 mg/kg of bicuculline in male or female LSxSS RIs.

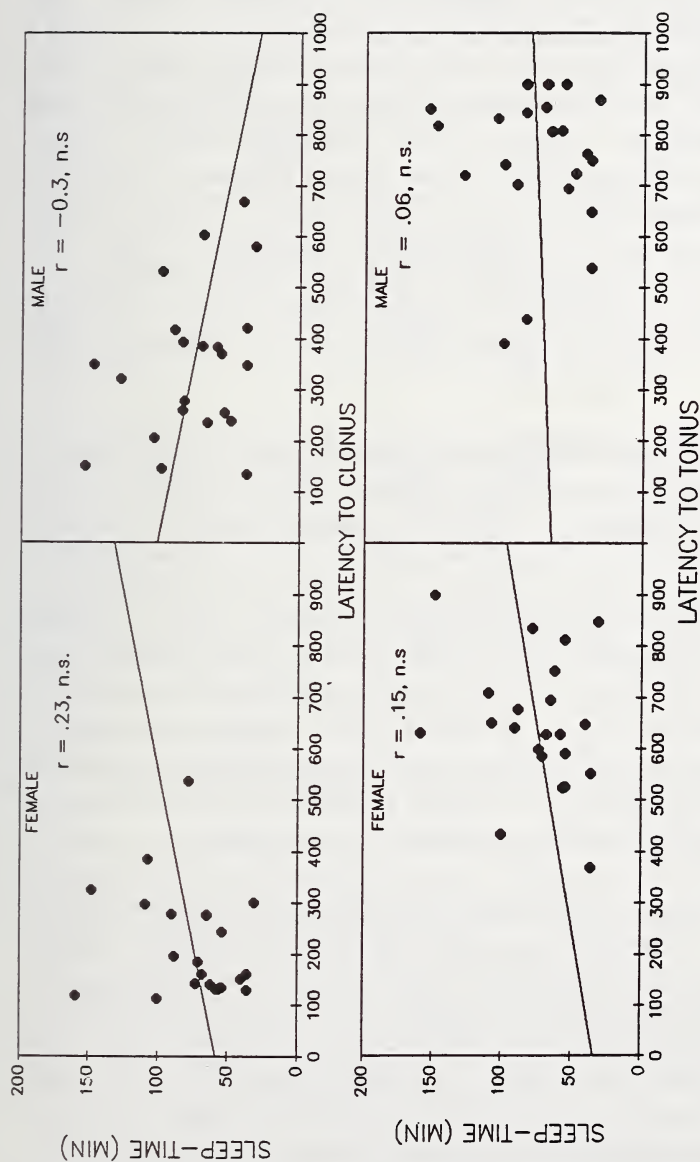


Figure 5.—Correlation of the mean duration of sleep time response after a 4.1 g/kg (20 percent [wt/vol] of ethanol in LSxSS RIs versus latency to clonus or tonus after 4.0 mg/kg of bicuculline in male and female mice.

seizure initiation and propagation. These pathways would be expected to be regulated by multiple genes, resulting in the behavioral response. The different susceptibilities among genetic stocks of mice may be at the receptor level, the chloride ionophore, or at other stages of seizure activity. At this time, we have not observed a consistent GABA receptor difference by using ^3H -GABA binding as a measure of the low-affinity GABA receptor. Likewise, Allan and Harris (1986) did not observe differences in muscimol binding at the higher affinity site of the GABA receptor. Recently, Peris et al. (personal communication) have observed a relationship between ^{35}S -*t*-butylbicyclopophosphorothionate (TBPS) binding in inferior colliculus and bicuculline seizure susceptibility in the RIs. This provides a promising candidate for at least one of the genes involved in regulating seizure threshold.

The lack of commonality between genes regulating seizure susceptibility and ethanol sensitivity is supported further by data generated by using another GABAergic seizure inducer, 3MP. This competitive inhibitor of GAD produces seizures in both LS and SS mice, but the pattern of susceptibility is opposite that observed with bicuculline. LS mice are more sensitive than SS mice (figure 6). Biochemical studies of GABA enhancement of benzodiazepine binding in LS, SS, and inbred mouse strains indicated that the variation in this binding parameter correlated positively with the latency to seizure production (Marley and Wehner 1987a). Whether this measure is correlated to susceptibility to other types of seizure inducers remains to be determined. On the basis of the opposite sensitivities of LS and SS mice to 3MP and bicuculline, it would appear that this is not the case.

Utilization of a variety of measurements for the various receptor subunits and the interactions of those subunits within the GABAergic receptor complex in specific brain regions will be necessary to provide clues to the complex regulation of seizure susceptibility to a variety of seizure inducers and other behavioral phenotypes. The bicuculline and 3MP seizure data support the conclusion that there is no genetic or mechanistic commonality between baseline seizure susceptibility to GABAergic agents and ethanol sensitivity. A more fruitful area of exploration may be the examination of sensitivity to ethanol withdrawal seizures in various inbred strains and selected lines of mice.

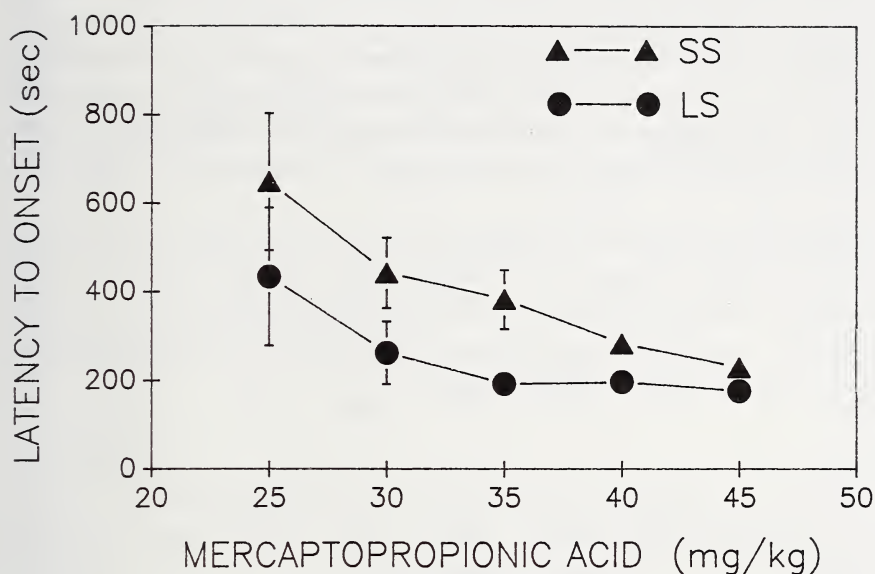


Figure 6.—Latency to onset of seizures induced by 3MP in female LS and SS mice. Each point represents the mean \pm SEM for six mice.

Anticonvulsant Effects of Benzodiazepines

As shown above, seizures can be differentially induced in LS and SS mice by manipulation of the GABAergic system. Occupation of the benzodiazepine receptor *in vivo* produces potent inhibition of seizure activity (Braestrup and Neilson 1986), and sensitivity to these anticonvulsant effects of benzodiazepine may vary according to genotype. As another indicator of actions at the receptor complex, we examined the anticonvulsant effects of two benzodiazepines, flurazepam and diazepam. To examine these effects, we used 3MP (15-55 mg/kg) and examined the increased latency to seizure after benzodiazepine pretreatment. In both LS and SS mice, the benzodiazepine flurazepam significantly increased the latency to seizures induced by 3MP [$F_{(4,74)}=35.2$, $P<0.001$] over a dose range of 1-6 mg/kg (figure 7). The SS mice, however, were more sensitive to this effect of

flurazepam such that the dose needed to increase the latency of onset to 600 s after a 55-mg/kg dose of 3MP was 2.42 ± 0.24 mg/kg, compared with 4.72 ± 0.23 mg/kg in LS mice. This degree of differential sensitivity was quantitatively different between LS and SS mice according to dose of 3MP, but the rank order was always qualitatively the same; i.e., SS mice were always of the more sensitive genotype. This indicates that sensitivities to 3MP and to the anticonvulsant actions of flurazepam are independently regulated.

Likewise, the anticonvulsant diazepam increased the latency to seizures in both populations [$F_{(5,62)}=3.2$, $P<0.001$]; again the SS mice were more sensitive than the LS mice to the effects of diazepam [$F_{(1,62)}=53.1$, $P<0.001$; figure 8]. The LS mice were not protected at a dose of 1.0 mg/kg, whereas the SS mice were protected at 0.4 mg/kg.

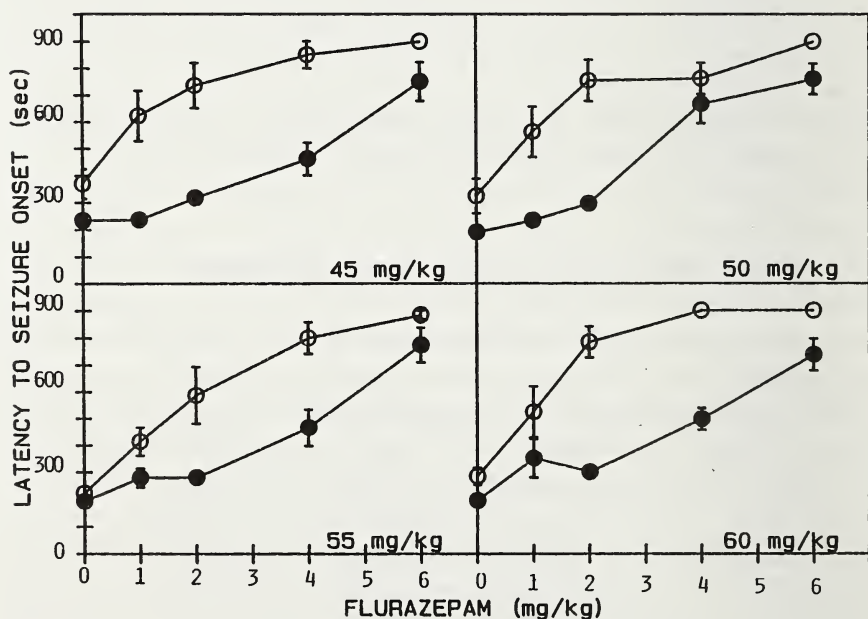


Figure 7.—Anticonvulsant actions of flurazepam against seizures induced by 3MP in female LS and SS mice. Each point represents the mean \pm SEM latency to the onset of seizures for 6-11 mice.

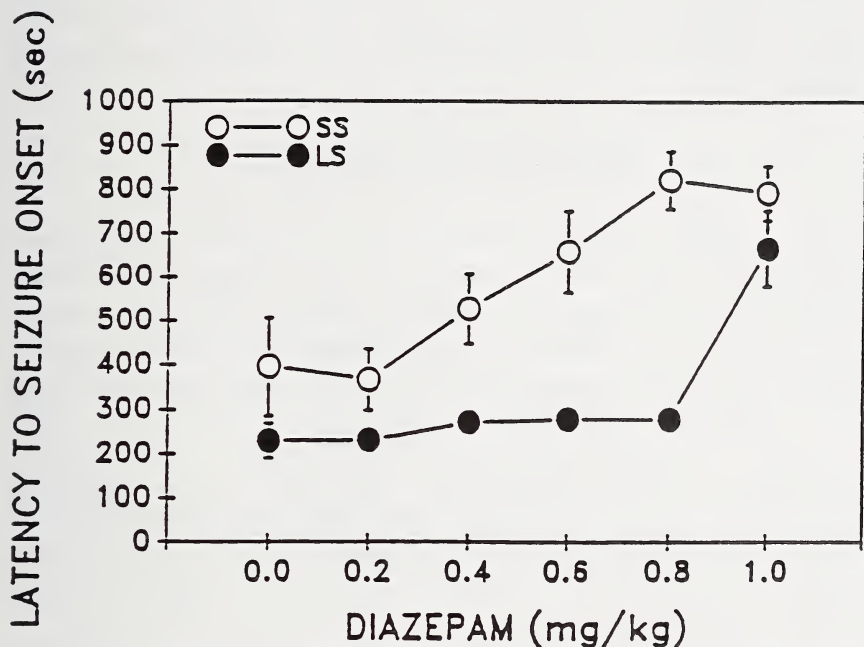


Figure 8.—Anticonvulsant actions of diazepam against seizures induced by 55 mg/kg of 3MP in female LS and SS mice. Each point represents the mean \pm SEM latency to the onset of seizures for five to seven mice.

There are two obvious conclusions from these studies on the anticonvulsant effects of benzodiazepines. First, these actions are produced at a much lower dose range than are the sedative-hypnotic and the hypothermic effects of benzodiazepines. Moreover, although LS mice are more sensitive to the effects of 3MP in the production of seizures, they are more resistant to the anticonvulsant effects of benzodiazepines.

These data suggest that there is not a simple relationship between the inhibition of presynaptic function, i.e., the inhibition of GAD by the competitive inhibitor 3MP, and the sensitivity to an anticonvulsant. It was somewhat surprising that no predictable pattern of benzodiazepine sensitivity was observed between LS and SS mice over the different behaviors. The rank order of sensitivity was dependent on behavior tested and dose range of the

benzodiazepine. We believe that an understanding of what appears to be a complex regulation of behavioral sensitivity lies in understanding specific regional distribution and heterogeneity of the GABA-benzodiazepine receptor complex.

Elevated Plus-Maze Responses

We have recently investigated another type of behavior mediated by benzodiazepines, the anxiolytic response. Such responses are generally produced by benzodiazepines in low doses but have been difficult to measure because of obvious differences between rodent and human behavior. Although proconflict paradigms have been widely used (Gardner and Peper 1982), we have not used these tests because they require water or food deprivation and administration of electrical shocks. Both of these manipulations could confound genetic comparisons because of varying animal weights and fat composition or differential pain thresholds. It has been proposed that measurements of anxiety in mice might best be performed by making use of exploration in a novel environment.

Such a test was designed by Pellow et al. (1985). This test consists of an elevated maze with two open and two closed arms. The natural response of the rodent is to enter more frequently, and spend more time in, the closed arms rather than the more threatening open arms. Extensive validation of this method by using benzodiazepines and measurement of the stress hormone, corticosterone, has been performed. One confound in the test is that some anxiolytics, such as ethanol, cause locomotor activation when administered in low doses. The interpretation of an anxiolytic versus an activating response can be sorted out by determining overall activity in all arms of the maze.

The elevated plus-maze was used to determine the responses to ethanol and diazepam in LS and SS mice. SS mice entered and spent more time in the open arms of the maze after ethanol injection [$F_{(5,99)}=5.95$, $P<0.001$; $F_{(5,99)}=81.9$, $P<0.001$] (figure 9). LS mice did not exhibit a significant change in behavior at any low dose of ethanol and became sedated at higher doses. It can be concluded that LS mice appear to be insensitive to the anxiolytic effects of ethanol in the low dose range. Measurements of total activity

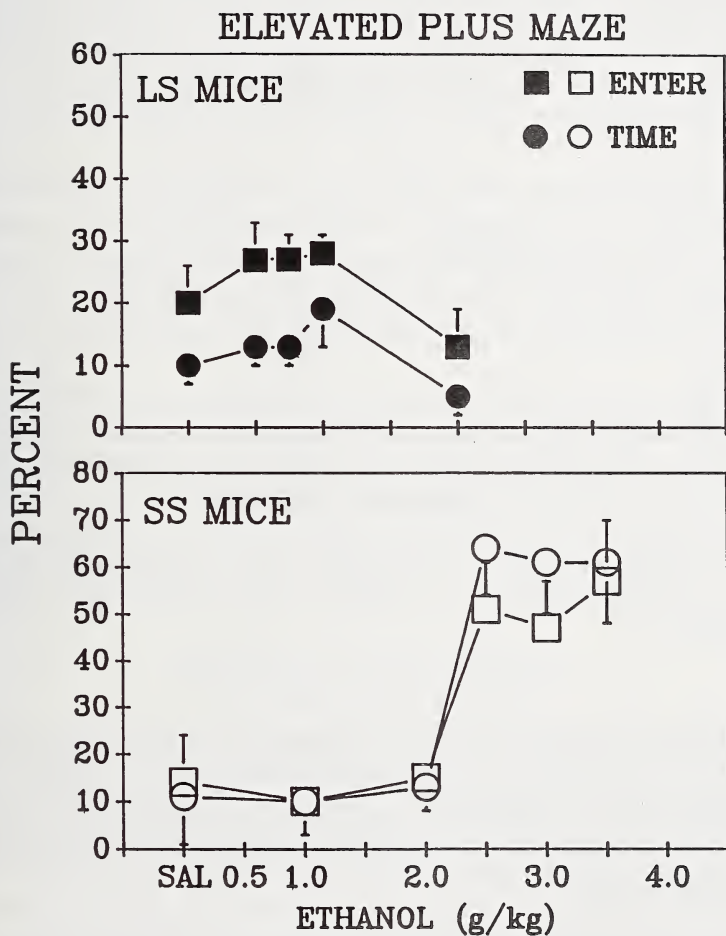


Figure 9.—Elevated plus-maze activity after various doses of ethanol in male δ and SS mice. Each point represents the mean \pm SEM for nine mice.

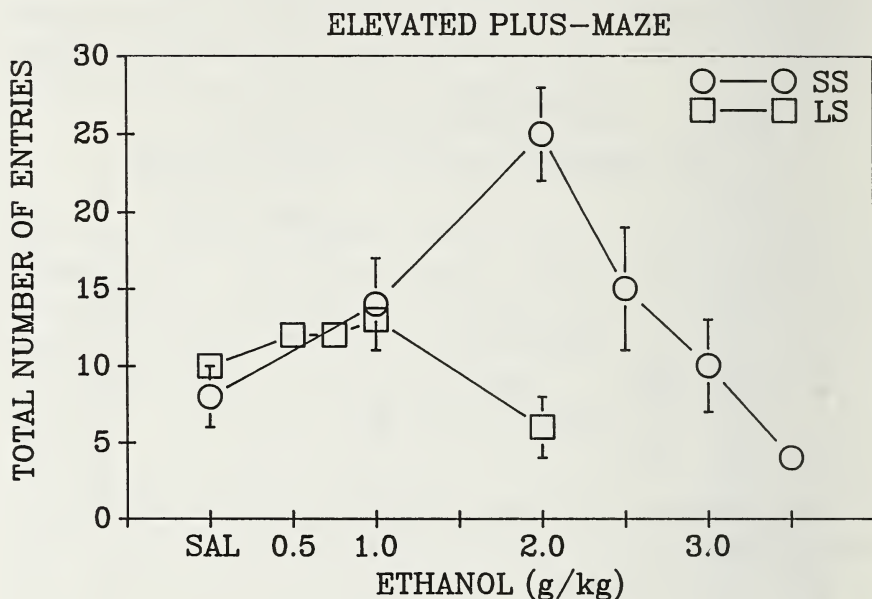


Figure 10.—Number of entries in the elevated plus-maze after various doses of ethanol in male LS and SS mice. Each point represents the mean \pm SEM for nine mice.

(figure 10) in the SS mice indicated that ethanol also had an activating effect [$F_{(5,59)}=6.51$, $P<0.001$], but post hoc analysis showed that this activation was observed only at the 2.0-g/kg dose ($P<0.01$) and not at doses higher than 2.0 g/kg. LS mice exhibited a decrease in activity at the 2.0 g/kg dose [$F_{(4,49)}=4.53$, $P<0.01$].

It appears that in SS mice some of the anxiolytic effect of ethanol might be due to behavioral activation. Studies are underway to examine ethanol's anxiolytic effects by other methods. Although this test appears attractive because of its simplicity, it may not be ideal for future studies of ethanol's anxiolytic actions in animals such as the LS mice that are extremely sensitive to ethanol's sedative effects because the anxiolytic range for ethanol is quite high (Durcan and Lister 1988).

For the purpose of comparison, the effects of diazepam in LS and SS mice were also examined by using the elevated plus-maze. After an acute exposure to diazepam (figure 11), LS mice spent more time in the open arms of the maze [$F_{(4,49)}=2.54$, $P<0.05$]. SS mice were insensitive to this effect of diazepam. As with ethanol, some of the effects of diazepam appeared to be due to behavioral activation, since the total activity in the LS mice was significantly increased above 1.0 mg/kg [$F_{(4,49)}=2.82$, $P<0.05$; figure 12].

A conservative interpretation of these data would suggest a lack of a true anxiolytic response to either ethanol or diazepam in LS and SS mice. However, Durcan and Lister (1988) have recently argued that the activating effects are observed only at limited doses and times after ethanol and therefore do not interfere with the anxiolytic effects observed at other doses.

We have found that manipulation of this test has been useful for induction of anxiety by restriction of the animals to the open arms of the maze for a defined period of time (see below). We are currently using this paradigm to examine the effects of ethanol and benzodiazepines. It may be that this test can be modified to provide a more useful measure of anxiogenic and anxiolytic responses.

Manipulation of the GABAergic Receptor System by Stress

Recent studies indicate that steroid hormones and their metabolites can modify GABAergic receptor function (Majewska 1987). The concept that steroids or their metabolites are endogenous modulators of the GABAergic system is provocative because many stressful situations elevate plasma levels of steroids, including the hormone corticosterone, in rodents. If this is the case, endogenous elevations of neural modulators could alter the state of GABAergic function before *in vitro* biochemical experiments are performed. This question is of potential interest to the field of ethanol research because the effects of ethanol in tissue preparations derived from living animals have at times been difficult to reproduce, resulting in some uncertainty in the literature. For example, the enhancement of benzodiazepine binding by ethanol has been the subject of some debate (Greenberg et al. 1984). We therefore have begun a series of experiments to demonstrate whether any of

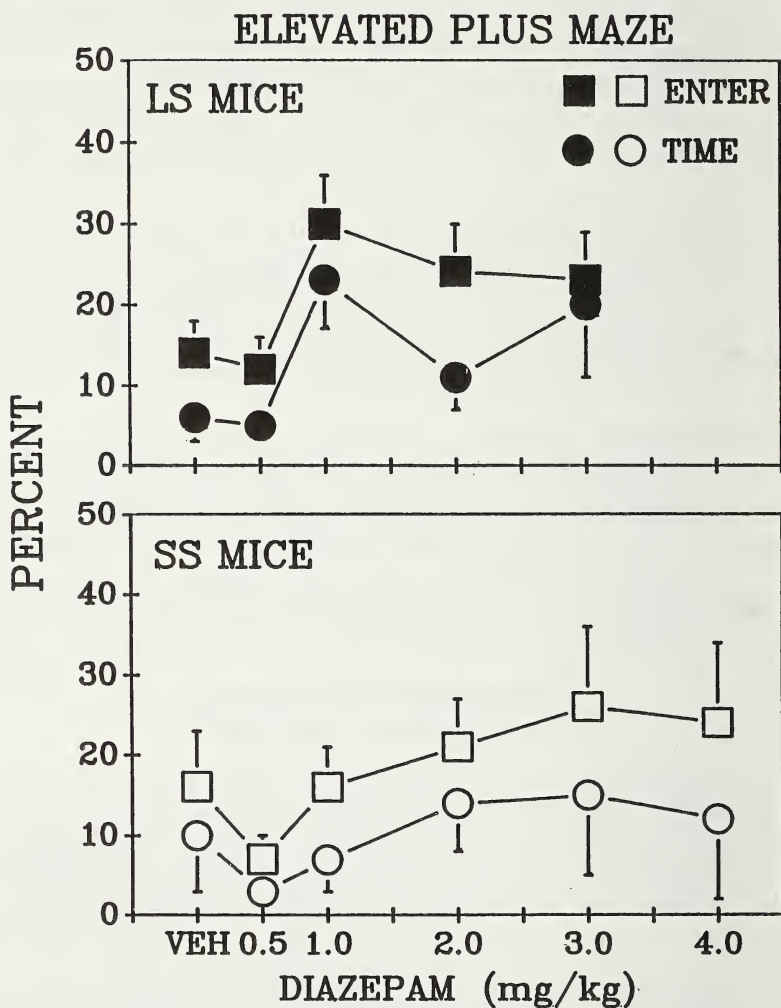


Figure 11.—Elevated plus-maze activity after various doses of diazepam in male LS and SS mice. Each point represents the mean \pm SEM for nine mice.

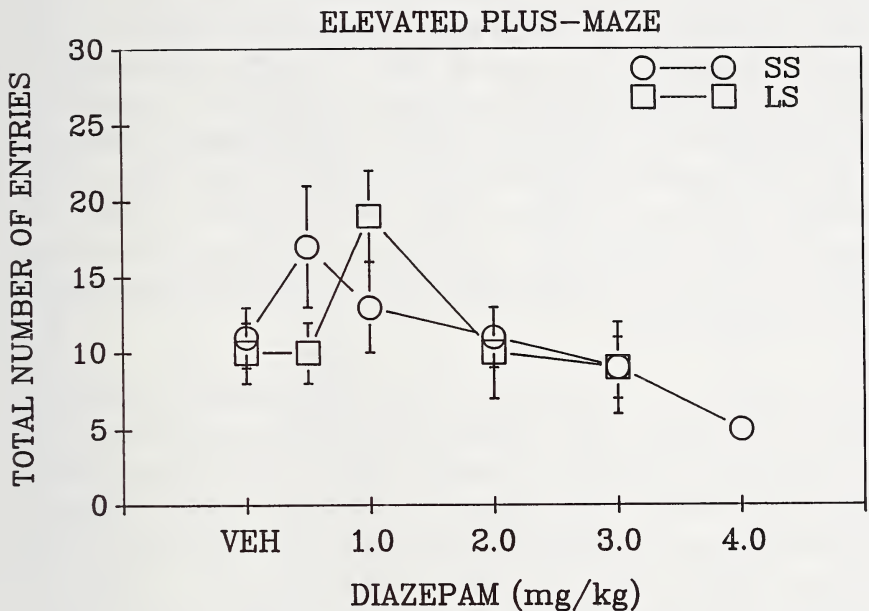


Figure 12.—Number of entries in the elevated plus-maze after various doses of diazepam. Each point represents the mean \pm SEM for nine mice.

ethanol's effects might be mediated via the known effects of ethanol on the adrenal-pituitary axis. An IP injection of ethanol produces an elevation in corticosterone release that is genotype dependent (Kakihana 1976). Our preliminary results are present here to provide evidence that in vitro assessment of GABAergic function must be carefully evaluated in terms of the specific assay conditions, as well as in terms of the environmental variables to which the animal was exposed before sacrifice (Martin and Wehner 1988).

Very simply, we have asked whether a stressed animal has altered GABAergic function because of steroid hormone release, using two behavioral manipulations that cause an elevation in corticosterone. In the first set of experiments, we used the elevated plus-maze for stress induction

by restricting LS and SS mice to the open arm of the maze. This environment causes an increase in corticosterone release (Pellow et al. 1985). After sequestration on the open arm, LS and SS mice were sacrificed and GABA enhancement of benzodiazepine binding was examined. The behavioral manipulation did have an effect on GABA enhancement of benzodiazepine binding, but the magnitude of the effect was genotype dependent (figure 13). After stress, SS mice showed a marked increase in GABA enhancement of ^3H -flunitrazepam binding in cortex [$F_{(1,49)}=102.2$, $P<0.001$], but LS mice showed a lesser effect [$F_{(1,49)}=5.7$, $P<0.05$]. A different picture emerged in the cerebellum. LS mice were dramatically affected [$F_{(1,49)}=105.8$, $P<0.001$], but SS mice were affected to a lesser degree [$F_{(1,49)}=18.1$, $P<0.001$].

In the second set of experiments, we used an IP injection of ethanol to elevate corticosterone release. Kakihana (1976) has shown that the elevation in corticosterone is genotype dependent, with LS mice producing higher levels than SS mice. An IP injection of ethanol at 2.5 g/kg produced a differential release of corticosterone in LS and SS mice (381 ± 10 ng/ml in SS mice and 570 ± 45 ng/ml in LS mice). This treatment also resulted in a change in GABA enhancement of benzodiazepine binding (figure 14), with a pattern similar to that observed with the elevated plus-maze used as a stressor. We have previously shown by using heat denaturation techniques that LS and SS mice differ in the molecular characteristics of cortical and cerebellar benzodiazepine receptors; therefore, it was not surprising that they were affected differently by these behavioral treatments.

To demonstrate that this alteration in coupling between the benzodiazepine receptor and the GABA receptor caused by stress is mediated via adrenal release, LS and SS mice were adrenalectomized and then tested 1 week later by an IP injection of ethanol (2.5 g/kg). Adrenalectomy reversed the stress effect on GABA enhancement in LS and SS cerebellum such that an injection of ethanol caused an increase in GABA enhancement in sham-operated animals but not in those that had been adrenalectomized (figure 15). Control saline-injected animals that do not show a profound increase in corticosterone release after the saline injection are unaffected by adrenalectomy. The reversal of the effects of ethanol would be consistent with the interpretation that some adrenal compound, perhaps corticosterone,

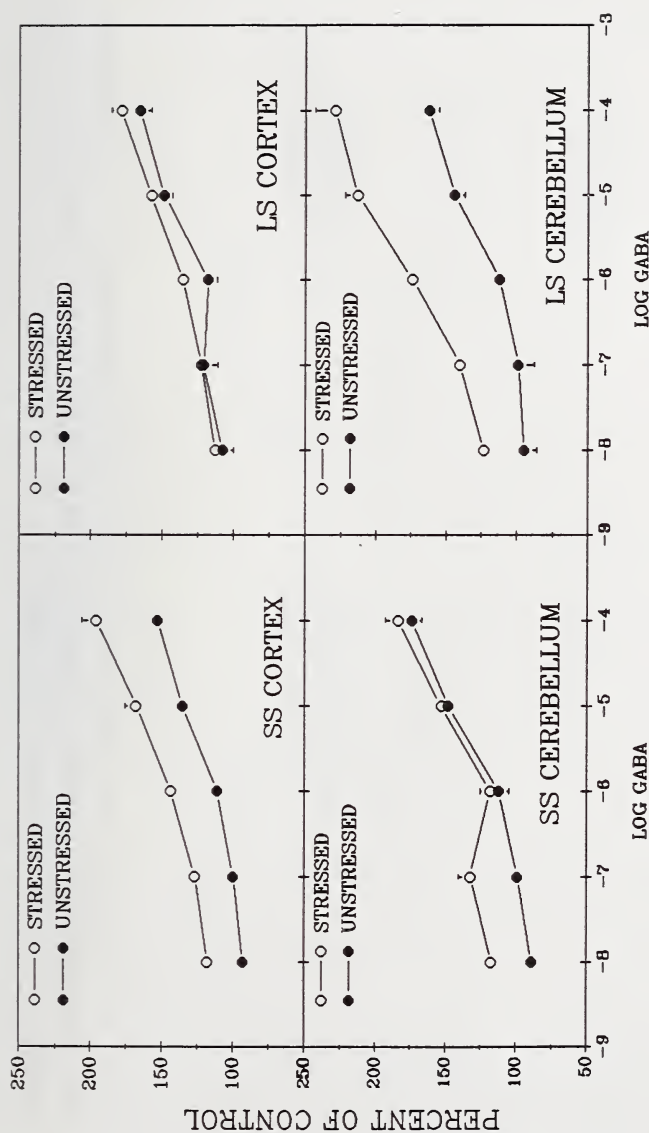


Figure 13.—Effect of stress on GABA enhancement of ^3H -flunitrazepam binding in cortical and cerebellar membranes prepared from LS and SS mice. Each point represents the mean \pm SEM of five experiments.

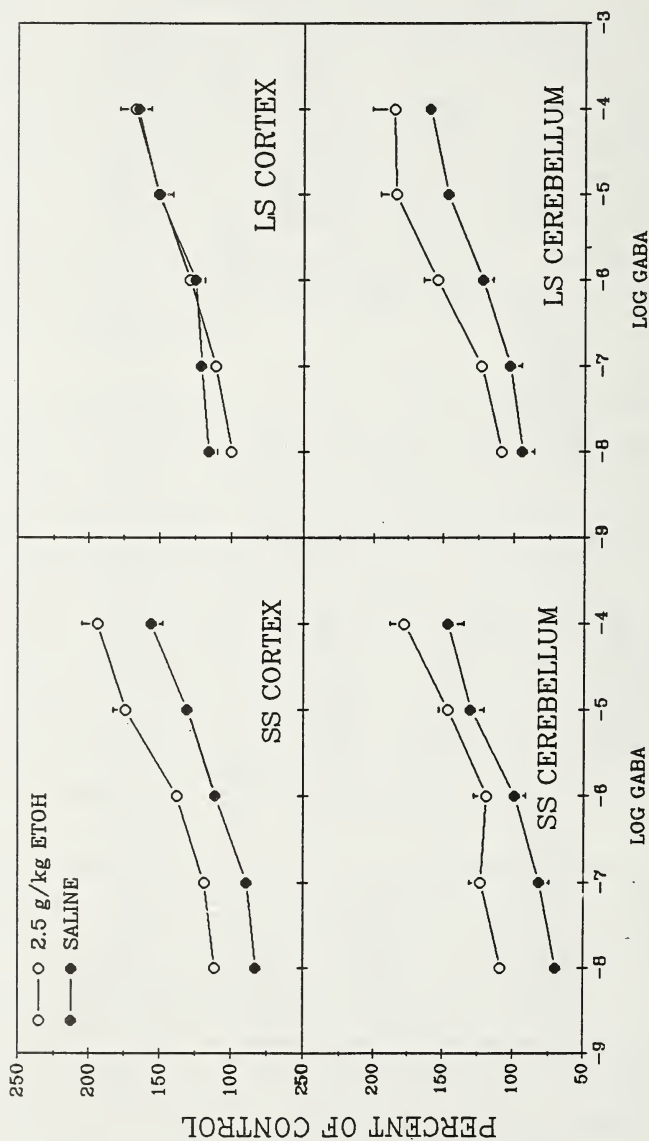


Figure 14.—Effect of an acute injection of ethanol on GABA enhancement of ^3H -flunitrazepam binding in cortical and cerebellar membranes prepared from LS and SS mice. Each point represents the mean \pm SEM of five experiments.

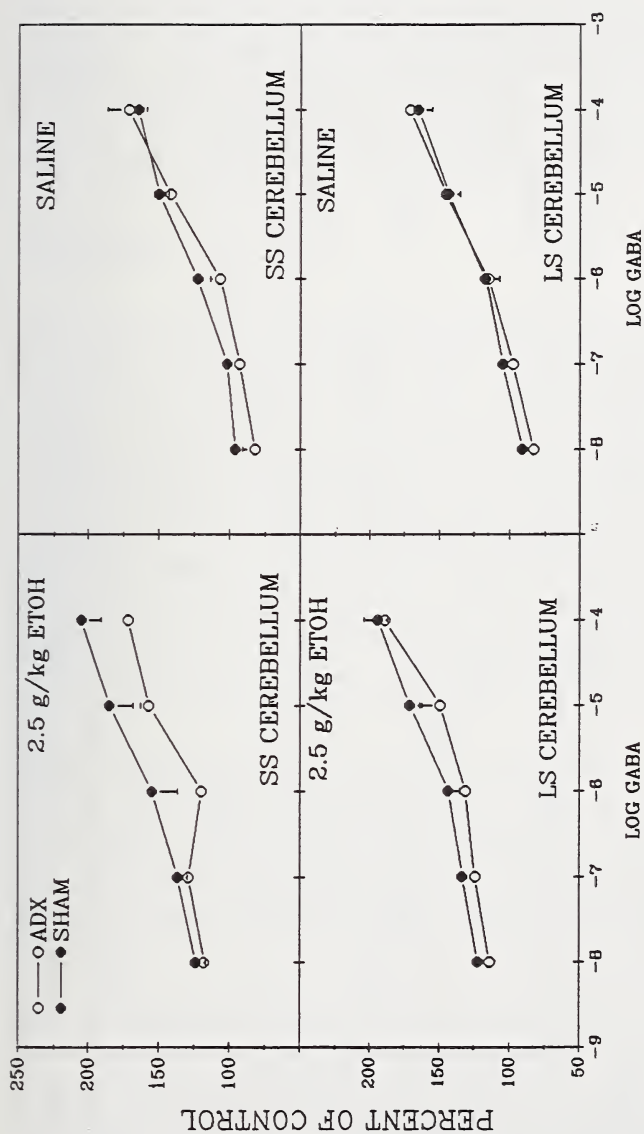


Figure 15.—Effect of an acute injection of ethanol on GABA enhancement of ³H-flunitrazepam binding in cerebellar membranes prepared from sham and adrenalectomized LS and SS mice. Each point represents the mean \pm SEM of five experiments.

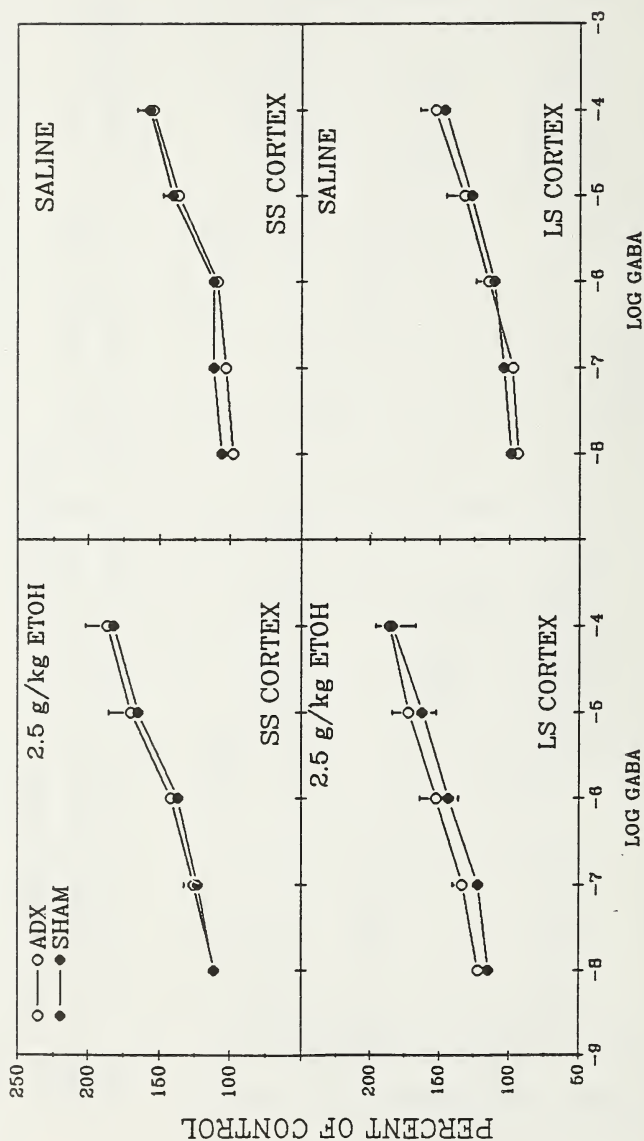


Figure 16.—Effect of an acute injection of ethanol on GABA enhancement of ³H-flunitrazepam binding in cortical membranes prepared from sham and adrenalectomized LS and SS mice. Each point represents the mean \pm SEM of five experiments.

alters the coupling of the benzodiazepine receptor with the GABA receptor. The same pattern of reversal was not observed in cortex (figure 16). Although the nature of the compounds producing these altered interactions is presently unclear, the results suggest that interpretation of GABAergic receptor function by *in vitro* experimentation should be done with caution and that care should be taken to provide a stress-free environment for animals used in biochemical experimentation.

Summary

The measurement of behavioral responses after manipulations of the GABAergic system has aided in sorting out those behaviors that appear to be under common genetic and mechanistic regulation with initial sensitivity to ethanol. Clearly, the sedative-hypnotic response to ethanol and benzodiazepines is the best candidate for such an association at this point because this effect has been supported by results from both behavioral and biochemical studies. On the other hand, it appears that seizure sensitivity to GABAergic agents does not reflect a single gene phenomenon or a shared genetic link with ethanol sensitivity. The common regulation of ethanol-induced and benzodiazepine-induced hypothermia does not look promising. Finally, it may be premature to draw conclusions concerning the anxiolytic actions of either ethanol or benzodiazepine.

Without question, pharmacogenetic approaches provide a powerful tool to examine complex relationships in the CNS and to evaluate drug responsiveness. The availability of the LSxSS RIs, Crabbe's selected lines of mice (Crabbe et al. this volume), and the benzodiazepine selected lines of Gallaher (Gallaher and Gionet 1988) will allow more definitive studies in the near future.

Ethanol is thought to mediate some of its actions via interactions with the γ -aminobutyric acid (GABA)ergic receptor complex. The question of whether various responses to ethanol and GABAergic agents are regulated by common mechanisms was investigated by using a pharmacogenetic approach. Long-sleep (LS) and short-sleep (SS) mice were screened on a variety of behaviors thought to be mediated by the GABAergic system. The results of

these studies indicated that the sedative-hypnotic response to benzodiazepines is similar to the LS and SS response to ethanol, suggesting that a common mechanism regulates this behavior. However, no commonalities were observed among benzodiazepine-induced hypothermia, 3-mercaptopropionic acid seizure susceptibility, anticonvulsant effects of benzodiazepines, or anxiolytic responses to ethanol. Although bicuculline-induced seizures appeared to be related to initial sensitivity to ethanol in LS and SS mice, a more exhaustive examination in 22 recombinant inbred strains of LS and SS mice showed no relationship between these two behaviors. The results of this study demonstrate that many behaviors related to the GABAergic system and responses related to ethanol sensitivity are not regulated by common genes or mechanisms.

References

- Allan, A.M.; Gallaher, E.J.; Gionet, S.E.; and Harris, R.A. Genetic selection for benzodiazepine ataxia produces functional changes in the gamma-aminobutyric acid receptor chloride channel complex. *Brain Res* 452:118-126, 1988a.
- Allan, A.M., and Harris, R.A. Gamma-aminobutyric acid and alcohol actions: Neurochemical studies of long sleep and short sleep mice. *Life Sci* 39:2005-2015, 1986.
- Allan, A.M.; Spuhler, K.P.; and Harris, R.A. Gamma-aminobutyric acid activated chloride channels: Relationship to genetic differences in ethanol sensitivity. *J Pharmacol Exp Ther* 244:866-870, 1988b.
- Braestrup, C., and Neilson, M. Benzodiazepine receptor binding in vivo and efficacy. In: Olson, R.W., and Ventor, J.N., eds. *Benzodiazepine/GABA Receptor and Chloride Channels: Structural and Functional Properties*. New York: Alan R. Liss, 1986.
- Burch, T.P., and Ticku, M.K. Ethanol enhances ³H-diazepam binding at the benzodiazepine-GABA-receptor-ionophore complex. *Eur J Pharmacol* 67:325-326, 1980.

- DeFries, J.C.; Wilson, J.R.; Erwin, V.G.; and Peterson, D.R. LSxSS recombinant inbred strains of mice: Initial characterization. *Alcohol Clin Exp Res* 13:196-200, 1989.
- Dudek, B.C.; Abbott, M.E.; and Phillips, T.E. Stimulant and depressant properties of sedative-hypnotics in mice selectively bred for differential sensitivity to ethanol. *Psychopharmacology* 82:46-51, 1984.
- Durcan, M.J., and Lister, R.G. Time course of ethanol's effects on locomotor activity, exploration, and anxiety in mice. *Psychopharmacology* 96:67-72, 1988.
- File, S.E. What can be learned from the effects of benzodiazepines on exploratory behavior? *Neurosci Biobehav Rev* 9:45-54, 1985.
- Freund, R.K.; Marley, R.J.; and Wehner, J.M. Differential sensitivities to seizure-inducing and electrophysiological effects of bicuculline in three mouse strains. *Brain Res Bull* 18:657-662, 1987.
- Frye, G.D., and Breese, G.R. GABAergic modulation of ethanol-induced motor impairment. *J Pharmacol Exp Ther* 223:750-756, 1982.
- Gallaher, E.J., and Gionet, S.E. Initial sensitivity and tolerance to ethanol in mice genetically selected for diazepam sensitivity. *Alcohol Clin Exp Res* 12:77-80, 1988.
- Gardner, C.R., and Peper, D.C. Effects of agents which enhance GABA-mediated neurotransmission on licking conflict in rats and exploration in mice. *Eur J Pharmacol* 83:25-33, 1982.
- Greenberg, D.A.; Cooper, E.C.; Gordon, A.; and Diamond, I. Ethanol and the gamma-aminobutyric acid-benzodiazepine receptor complex. *J Neurochem* 42:1062-1068, 1984.
- Haefely, W.P.; Ploc, P.; Pieri, L; Schaffner, R.; and Laurent, J.P. Neuropharmacology of benzodiazepine synaptic mechanisms and neural basis of action. In: Costa, E., ed. *The Benzodiazepines: Synaptic Mechanisms and Neural Basis of Action*. New York: Raven Press, 1983. pp. 21-67.

- Harris, R.A., and Allan, A.M. Genetic differences in coupling of benzodiazepine receptors to chloride channels. *Brain Res* 490:26-32, 1989.
- Howerton, T.C.; O'Connor, M.J.; and Collins, A.C. Differential effects of long-chain alcohols in long- and short-sleep mice. *Psychopharmacology* 79:313-317, 1983.
- Kakihana, R. Adrenocortical function in mice selectively bred for different sensitivity to ethanol. *Life Sci* 18:1131-1138, 1976.
- Le, A.D.; Khanna, J.M.; Kalant, H.; and Gross, F. Tolerance to and cross-tolerance among ethanol, pentobarbital, and chlordiazepoxide. *Pharmacol Biochem Behav* 24:93-98, 1986.
- Liljequist, S., and Engel, J.A. Effects of GABAergic agonists and antagonists on various ethanol-induced behavioral changes. *Psychopharmacology* 78:71-75, 1982.
- Lister, R.G. Interactions of three benzodiazepines receptor inverse agonists with ethanol in a plus-maze test of anxiety. *Pharmacol Biochem Behav* 30:701-706, 1988.
- Majewska, M. Steroid and brain activity. *Biochem Pharmacol* 36:3781-3788, 1987.
- Marley, R.J.; Freund, R.K.; and Wehner, J.M. Differential response to flurazepam in long-sleep and short-sleep mice. *Pharmacol Biochem Behav* 31:453-458, 1988.
- Marley, R.J.; Miner, L.L.; Wehner, J.M.; and Collins, A.C. Differential effects of central nervous system depressants in long-sleep and short-sleep mice. *J Pharmacol Exp Ther* 238:1028-1033, 1986.
- Marley, R.J.; Stinchcomb, A.; and Wehner, J.M. Further characterization of benzodiazepine receptor differences in long-sleep and short-sleep mice. *Life Sci* 43:1223-1231, 1988.

- Marley, R.J.; and Wehner, J.M. GABA enhancement of flunitrazepam binding in mice selectively bred for differential sensitivity to ethanol. *Alcohol Drug Res* 7:25-32, 1987a.
- Marley, R.J., and Wehner, J.M. Correlation between the enhancement of flunitrazepam binding of GABA and seizure susceptibility in mice. *Life Sci* 40:2215-2224, 1987b.
- Martin, B.J., and Wehner, J.M. Influence of genotype on nicotine-induced increases of plasma corticosterone in mice as a result of acute nicotine pretreatment. *Pharmacol Biochem Behav* 30:1065-1070, 1988.
- Martin, B.J., and Wehner, J.M. Stress hormones, ethanol and GABA interactions in LS and SS mice. *Soc Neurosci Abstr* 14:346, 1988.
- Martz, A.R.; Deitrich, R.A.; and Harris, R.A. Behavioral evidence for the involvement of gamma-aminobutyric acid in the actions of ethanol. *Eur J Pharmacol* 89:53-62, 1983.
- McCleary, G.E., and Kakihana, K. Selective breeding for ethanol sensitivity: SS and LS mice. In: McCleary, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*, DHHS Pub. No. ADM (81-1113). Washington, DC: Supt. of Docs., U.S. Gov. Print. Off., 1981. pp. 147-159.
- McIntyre, T.D.; and Alpern, H.P. Thiopental, phenobarbital, and chlordiazepoxide induce the same differences in narcotic reaction as ethanol in long-sleep and short-sleep selectively bred mice. *Pharmacol Biochem Behav* 24:895-898, 1986.
- Miller, L.; Greenblatt, D.J.; Barnhill, J.G.; and Shader, R.J. Differential modulation of benzodiazepine receptor binding by ethanol in LS and SS mice. *Pharmacol Biochem Behav* 29:471-477, 1988.
- Pellow, S.; Chopin, P.; File, S.E.; and Briley, G. Validation of open-closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods* 14:149-167, 1985.

- Phillips, T.J., and Dudek, B.C. Bicuculline-induced seizures in mice which differ in sensitivity to ethanol. *Soc Neurosci Abstr* 9:1240, 1983.
- Sanders, B. Sensitivity to low doses of ethanol and pentobarbital in mice selected for sensitivity to hypnotic doses of ethanol. *J Comp Physiol Psychol* 90:394-398, 1976.
- Sorenson, S.T.; Palmer, M.; Dunwiddie, T.; and Hoffer, B. Electrophysiological correlates of ethanol-induced sedation in differentially sensitive lines of mice. *Science* 210:1143-1145, 1980.
- Stinchcomb, A.; Bowers, B.J.; and Wehner, J.M. The effects of ethanol and Ro 15-4513 on elevated plus maze and rotorod performance in long sleep and short sleep mice. *Alcohol* (in press).
- Suzdak, P.D.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. Ethanol stimulates gamma-aminobutyric acid receptor-mediated chloride transport in rat brain synaptoneurosomes. *Proc Natl Acad Sci USA* 83:4071-4075, 1986.
- Ticku, M.K.; Lowrimore, P.; and Lehoullier, P. Ethanol enhances GABA-induced ^{36}Cl -influx in primary spinal cord cultured neurons. *Brain Res Bull* 17:123-126, 1986.

Discussion

VOGEL: I am glad that I am not the only one to emphasize the importance of stress in the actions of ethanol. In your experiment, you show that you can get completely different results if the animal is stressed or resting. Of course, when you remove the adrenals, you remove the corticosteroid and the catecholamine source. In stress, the levels of corticosteroids increase 3- to 4-fold, whereas the levels of epinephrine increases 10- to 20-fold. Is it steroid, epinephrine, or both?

WEHNER: The reason that I reported steroid data is because of the studies that show that steroids can enhance GABA function. We do not know that it is the steroids until we do more definitive replacement studies in adrenalectomized mice.

SIGGINS: In spite of my bias against the GABA-ethanol story, I should say that Don Woodward's group has also had a problem in showing ethanol enhancement of GABAergic inhibition in the cerebellum. He recently reported in a Neuroscience Society abstract that he does see a small effect and now thinks that it may be diurnal. He thinks this may relate to the effects of steroids on the GABA system. The other problem is using anesthetized animals. Even in the slice preparation, we don't know if we have washed out the anesthetic or steroids completely or if there are residual effects.

Does the Benzodiazepine- γ -Aminobutyric Acid Receptor- Chloride Ionophore Complex Mediate Initial Sensitivity to Ethanol?: Studies Using Long- Sleep (LS) and Short-Sleep (SS) Mice¹

Todd D. McIntyre, Ramon Trullas, and Phil Skolnick²

Introduction

The pathology of alcoholism has recently engendered a considerable volume of research, much of it focused on the development and analysis of animal models of the human condition. These models have provided important insights into such contributing factors as alcohol preference, initial sensitivity, and tolerance. One of the most widely used methods for developing animal models of alcoholism is selective breeding. For example, rodent lines have been bidirectionally bred for alcohol preference (Eriksson 1968; Li and Lumeng 1977), first-dose sensitivity (McClearn and Kakahana 1973; Riley et

¹We would like to thank Eugene Thomas and his staff for providing the LS and SS selected lines used in this research.

²Laboratory of Neuroscience, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.

al. 1976; Eriksson and Rusi 1981), hypothermic response (Crabbe et al. 1987), and severity of withdrawal-induced seizures (Wilson et al. 1984; Crabbe et al. 1985).

Selective breeding involves examining a heterogeneous population for the trait of interest and systematically mating males and females of "high" phenotypic value as well as males and females of "low" phenotypic value. The rate of divergence of the mean phenotypic values for these newly selected populations is a function of the degree of heritability of the trait and the amount of selection pressure applied (McClern 1981). Thus, with succeeding generations, each line has a corresponding increase in the frequency of these "high" and "low" alleles at all relevant loci. Eventually, all genes influencing the selected trait should approach homozygosity. Although this phenomenon should apply to all relevant loci regardless of the degree of impact on the selected trait, it can be constrained somewhat by other factors, including natural selection (Roberts 1981). All loci that are not relevant to expression of the selected trait should continue to be heterozygous, although many important loci are homozygous before and after selection (Crabbe et al. 1987). This latter point indirectly addresses an issue of fundamental importance to the formulation of subsequent hypotheses via the selective breeding procedure. That is, are behavioral and biochemical traits that are found to be correlated with the selected phenotype a result of the pleiotropic effects of one or more intentionally selected genes, or do they result from unintended homozygosity at loci that are not involved in expressing the selected trait? Moreover, even if the two phenotypes are actually linked, the correlated phenotype may not be relevant to the physiologic process being investigated. These issues, as they pertain to the hypothesis under consideration, will be addressed further at the conclusion.

Selective Breeding for Initial Alcohol Sensitivity

After initial indications that sleep times were markedly different for C57/BL and BALB/c mice after an intraperitoneal injection of ethanol (Kakihana et al. 1966), a selection study was initiated whereby a heterogeneous (HS) population of mice were examined for initial hypnotic sensitivities to 3.3 g/kg of ethanol (McClern and Kakihana 1973). These selected lines, designated long sleep (LS) and short sleep (SS), exhibited no overlap in sleep times by

the 16th selected generation. Initial studies indicated no differences between lines in blood ethanol disappearance rates or activity of hepatic alcohol and aldehyde dehydrogenases (Heston et al. 1974). In addition, blood ethanol concentrations at the time the righting reflex returned were 1.65 times higher in SS mice than in LS mice (Erwin et al. 1976), clearly indicating a genetically selected central nervous system (CNS) difference in hypnotic reaction to ethanol.

Pharmacologic Responses of the LS and SS Mouse Lines

Subsequent studies have indicated that the LS and SS selected lines are similarly differentiated by other CNS sedatives-hypnotics. Thus, differential sensitivities to anesthetics (Koblin and Deady 1981), barbiturates (Dudek and Phillips 1983; McIntyre and Alpern 1983; Alpern and McIntyre 1985, 1986; Marley et al. 1986), benzodiazepines (McIntyre and Alpern 1983, 1986a), and depressants such as 1-phenylisopropyl adenosine (Dunwiddie and Proctor 1984) and baclofen (Martz et al. 1983) have also been reported between the LS and SS lines. Furthermore, convulsants such as bicuculline, caffeine, picrotoxin, pentylenetetrazol, flurothyl, and 3-carbomethoxy- β -carboline also markedly distinguish (but in an opposite direction) these lines (Greer and Alpern 1977; Phillips and Dudek 1983; McIntyre and Alpern 1986b). These results suggested that the selection program may not have specifically selected for either ethanol-induced hypnosis or general motor impairment (McIntyre and Alpern 1985). This is an important distinction, since these results parallel findings in similar selection programs involving both other alcohol-related behaviors (Riley et al. 1978, 1979; Malila 1978; Hellevuo et al. 1987) and initial sensitivity to benzodiazepines (Allan et al. 1988).

The Benzodiazepine-GABA Receptor Complex

Recently, a number of converging lines of evidence (Skolnick and Paul 1988; Squires 1988) have indicated that many of the convulsants and depressants which differentiate the LS and SS selected lines may exert their primary effects via perturbation of the benzodiazepine- γ -aminobutyric acid (GABA) receptor-chloride ionophore complex ("supramolecular complex"; Tallman et al. 1980). Thus far, however, several attempts at establishing differences

between the LS and SS lines on one or more of the multiple allosteric recognition sites on the supramolecular complex have been inconclusive. For instance, it has been reported that neither the K_d nor B_{\max} for the radioligand [^3H]flunitrazepam (benzodiazepine receptor) or [^3H]muscimol (GABA receptor) differs between the two lines (Allan and Harris 1986; Marley and Wehner 1987). However, by use of an *in vivo* technique, [^3H]Ro 15-1788 binding was found to be markedly higher in the cortex and hippocampus of LS mice compared with SS mice. This increased binding was attributed to a higher B_{\max} rather than any significant difference in apparent affinity (Miller et. al. 1988). It should be noted, however, that another report (Goeders et al. 1988) has found that many different classes of psychopharmacologic agents (e.g., neuroleptics) nonspecifically modulate *in vivo* [^3H]Ro 15-1788 binding, which suggests that these results should be interpreted with caution.

In addition to the recognition site characteristics of the constituent subunits on the supramolecular complex, the allosteric interactions of benzodiazepine and GABA receptors have been assessed in these lines. Thus, both the potency and efficacy of GABA to allosterically enhance [^3H]flunitrazepam binding have been shown to be significantly greater in SS than in LS mice (Marley and Wehner 1987). These latter findings, in conjunction with those concerning binding characteristics, suggest that only modest (if any) differences exist between the LS and SS lines in the recognition site qualities of the benzodiazepine and GABA receptors. In contrast, further examination of the coupling among the individual subunits of the supramolecular complex may offer some insight into the unique pharmacogenetic profile of these two mouse lines.

Biophysical Differences in the Supramolecular Complex of LS and SS Mice

In an ongoing investigation of the biophysical properties of the α (benzodiazepine) and β (GABA) subunits of the supramolecular complex, we examined the thermal stability of benzodiazepine receptors in the LS and SS lines. A time-dependent reduction in [^3H]flunitrazepam binding was observed in the cortex and cerebellum of both lines after heat inactivation at 55 °C (figure 14). Cortical benzodiazepine receptors were inactivated at a 54

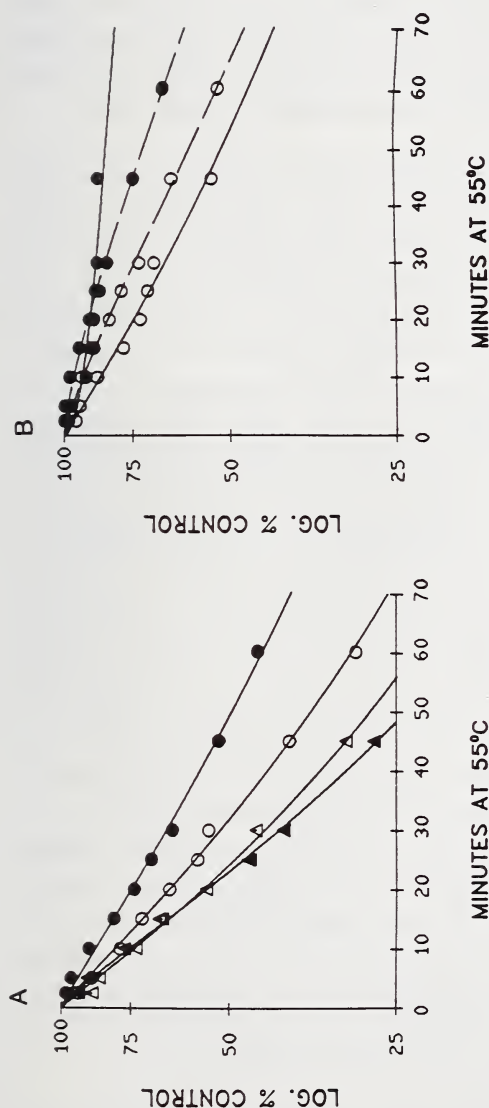


Figure 1.—Time course of thermal inactivation of [3 H]flunitrazepam binding to LS and SS cortical and cerebellar membranes. [3 H]flunitrazepam binding was determined in well-washed cortical and cerebellar membranes from LS and SS mice that had been immersed in a 55°C bath for 2.5–60 min before assay (see McIntyre et al. 1988 for experimental details). The radioligand concentration in these experiments was ≈ 1.6 nM. Data illustrated are from representative experiments repeated at least three times. The $t_{1/2}$ values were estimated by iterative fitting to the function $[f(x)] = 100 - (100/1 + (B^c/X^c))$, where B is $t_{1/2}$ and c is slope) on MLAB (NIH). Two-way analysis of variance revealed that there was a statistically significant effect of both mouse line [$F_{(1,10)} = 21.06, P < 0.001$] and GABA [$F_{(1,10)} = 66.04, P < 0.001$] on thermal inactivation, whereas no significant interaction was found. Post hoc analysis revealed a significant difference ($P < 0.05$) in the $t_{1/2}$ between these lines in both the presence and absence of GABA. Values: LS $t_{1/2} = 32.1 \pm 2.0$ min; SS $t_{1/2} = 49.5 \pm 5.7$ min. In the presence of GABA, LS $t_{1/2} = 65.8 \pm 2.9$ min, and SS $t_{1/2} = 92.7 \pm 6.8$ min. (A) Open symbols, LS; closed symbols, SS; circles, cortex; triangles, cerebellum. (B) Filled circles, SS; open circles, LS; dashed lines, inactivated in the presence of GABA (1.67 μ M); solid lines, inactivated in the presence of 0.5 μ M GABA and 200 mM NaCl.

percent slower rate in SS mice than in LS mice. Although cerebellar benzodiazepine receptors inactivated at a faster rate than cortical receptors, no line differences were evident in this tissue (figure 1A). In agreement with previous reports (Gavish and Snyder 1980), both GABA (1.67 μ M) and NaCl (200 mM) attenuated the rate of heat inactivation, while the overall differences between the LS and SS lines were maintained (figure 1B).

Since the differences in thermal inactivation suggested the presence of other differences in the biophysical properties of the supramolecular complex, GABA-enhanced [3 H]flunitrazepam binding was examined in these lines at 0 and 37 $^{\circ}$ C (figure 2B). As previously reported (Marley and Wehner 1987), GABA enhanced [3 H]flunitrazepam binding with greater potency and efficacy in cortical tissue of SS than of LS mice when assayed at 37 $^{\circ}$ C. However, when GABA-enhanced [3 H]flunitrazepam binding was assayed at 0 $^{\circ}$ C, the potency of GABA was higher in LS than SS membranes ($X + \text{SEM} = 0.26 \pm 0.03$ μ M versus 0.52 ± 0.07 μ M; $P < 0.05$). This temperature-dependent reversal in the potency of GABA is attributable to the potency of GABA not changing significantly in SS mice as the incubation temperature was increased from 0 to 37 $^{\circ}$ C, whereas a 300 percent decrease in the potency of GABA was observed in LS mice as the temperature was increased (figure 2A). Marley and Wehner (1986) also reported that there were no differences between LS and SS mice in either the K_d or B_{max} for [3 H]flunitrazepam binding when assayed at 37 $^{\circ}$ C. However there was a regional difference in [3 H]flunitrazepam binding, with LS mice having a twofold greater level of binding in the midbrain than SS mice. When assays were performed at 0 $^{\circ}$ C, similar results were found. Thus, when cortical tissue was incubated at 0 $^{\circ}$ C, an expected increase in apparent affinity of [3 H]flunitrazepam was obtained (Speth et al. 1978), but as reported at 37 $^{\circ}$ C, discernible differences between lines were not evident (table 1). The only apparent regional differences in specific binding between lines when assayed at 0 $^{\circ}$ C were in the midbrain (table 1 legend). Similarly, when the benzodiazepine partial inverse agonist [3 H]Ro 15-4513 (Sudzak et al. 1986) was used as a radioligand (0 $^{\circ}$ C), no significant differences for K_d or B_{max} were obtained (table 1).

Taken together, these findings indicate the presence of robust differences between these lines in the biophysical properties of the benzodiazepine-GABA receptor complex, without appreciable differences in the recognition

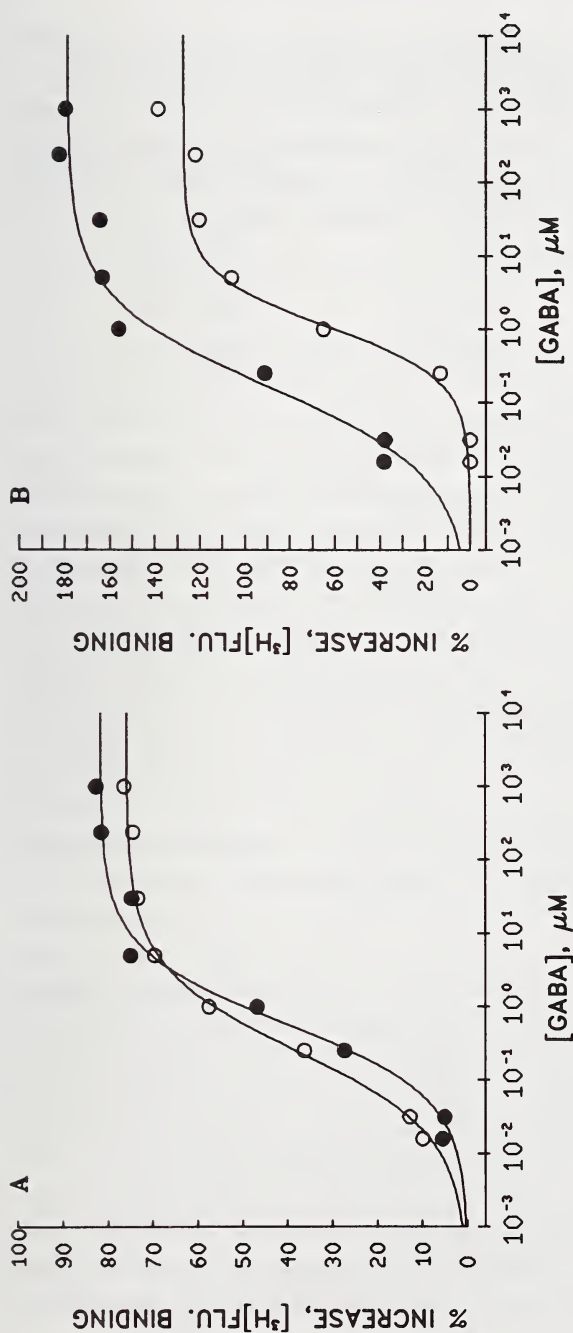


Figure 2.—GABA-enhanced [^3H]flunitrazepam binding in LS and SS cortical membranes: effect of incubation temperature. [^3H]flunitrazepam binding to well-washed cortical membranes from LS (o) and SS (•) mice was assayed at 0 °C (A) and 37 °C (B) as described previously (McIntyre et al. 1988). These representative curves were estimated by iterative fitting to a sigmoidal function $\{f(X) = A / (1 + (B^C/X^C))\}$, where A is maximum enhancement (E_{max} in percent increase over basal [^3H]flunitrazepam binding), B is concentration of GABA producing half-maximum enhancement (EC_{50} in micromolar concentration), and C is slope; modified from Spencer and Traber 1987) on MLAB (NIH). Mean \pm SEM values: 0 °C = LS, $EC_{50} = 0.26 \pm 0.03$, $E_{\text{max}} = 71.1 \pm 12.9$; SS, $EC_{50} = 0.52 \pm 0.07$, $E_{\text{max}} = 72.0 \pm 8.8$; 37 °C = LS, $EC_{50} = 0.79 \pm 0.13$, $E_{\text{max}} = 111.0 \pm 6.1$; SS, $EC_{50} = 0.42 \pm 0.11$, $E_{\text{max}} = 161.4 \pm 7.8$. Duncan's post hoc test revealed a significant difference between lines in efficacy only at 37 °C ($P < 0.05$). Similarly for potency: a significant difference between temperatures for LS but not for SS membranes and a significant difference between lines at both temperatures ($P < 0.05$).

Table 1.— K_d and B_{\max} values for [3 H]Ro 15-4513 and [3 H]flunitrazepam binding to well-washed cortical membranes from LS and SS mice^a

Experiment	Line	K_d (nM)	B_{\max} (fmol/mg of protein)
A	LS	2.7 ± 0.1	$3,867 \pm 179$
	SS	2.9 ± 0.1	$3,743 \pm 184$
B	LS	1.2 ± 0.8	$2,437 \pm 123$
	SS	1.2 ± 0.2	$2,386 \pm 74$

^aAssays were performed as described previously (McIntyre et al. 1988). Mice were housed in a quiet room for 2 hr before sacrifice, and only one animal per cage was removed for each experiment to minimize the effects of stress (Trullas et al. 1987). All assays were conducted at 0 °C. Data were obtained from Scatchard plots (correlation coefficients > 0.985) and represent the mean \pm standard error of the mean (SEM) of five animals for [3 H]Ro 15-4513 (A) binding and four animals for [3 H]flunitrazepam (B) binding. Assays contained 100 μ g of protein. Eight radioligand concentrations (0.4–20 nM) were used for [3 H]flunitrazepam binding, and seven concentrations (0.25–16 nM) were used for [3 H]Ro 15-4513 binding. Specific binding of [3 H]flunitrazepam (0.7–1.0 nM) in cerebellum, hippocampus, midbrain, and striatum revealed significant differences between lines only in the midbrain ($X \pm \text{SEM} = 646 \pm 29$ fmol/mg of protein for LS and 579 ± 39 fmol/mg of protein for SS; $t = 4.55$, degrees of freedom = 5, $P < 0.01$).

site qualities of its constituent subunits. A possible explanation is that the membrane-spanning domains of these receptors (Schofield et al. 1987) could be sufficiently different in the LS and SS lines to produce the results reported here. Since a previous study using electron spin resonance has shown that synaptosomal and erythrocyte membranes from LS mice are significantly more sensitive than SS membranes to the disordering effects of ethanol (Goldstein et al. 1982), a difference in membrane composition offers a plausible explanation for the temperature-dependant findings cited above. However, no differences were evident between lines in the rate of thermal (55 °C) inactivation of forebrain acetylcholine receptors using the muscarinic

antagonist [^3H]QNB ($X \pm \text{SEM } t_{1/2} = 15.4 \pm 1.0$ min for LS and 14.2 ± 0.8 min for SS). This latter finding suggests that the aforementioned biophysical differences may not be related to a general membrane property but rather are associated with some specific aspect of the supramolecular complex. This conclusion is supported by recent findings (Harris et al. 1988) demonstrating that after lipid extraction, fluorescence assays of liposomes no longer detect a difference between lines in membrane order, which suggests that some protein or lipid-protein interaction within the membrane is essential.

In addition, no significant differences in the rate of thermal inactivation of cerebellar benzodiazepine receptors were found. This observation provides further evidence that the differences in the biophysical properties of the supramolecular complex are specific and may be confined to a benzodiazepine receptor subtype (Lippa et al. 1979). Whereas several investigators (Sorensen et al. 1980, 1981; Allan and Harris 1986) have reported electrophysiologic and biochemical differences between LS and SS cerebella in response to ethanol, Palmer et al. (1984) demonstrated that neonatal cerebellectomy does not reduce the magnitude of the difference in hypnotic response to ethanol between these lines. Although this finding does not preclude the cerebellum from a role in mediating certain effects of ethanol (Palmer et al. 1987), it suggests that the cerebellum may not be an essential locus for mediating LS-SS differences.

The robust differences in thermal inactivation rates of cortical but not cerebellar benzodiazepine receptors in these lines may reflect the presence of different proportions of benzodiazepine receptor subtypes. Whereas initial reports had indicated only a single homogenous binding site for benzodiazepines (Mohler and Okada 1977; Squires and Braestrup 1977), subsequent reports have suggested the existence of two heterogenous benzodiazepine receptors (Lippa et al. 1979; Klepner et al. 1979); Squires et al. 1979; Sieghart and Karobath 1980). Since the triazolopyradazine CL 218,872 has been shown to distinguish two benzodiazepine receptor subtypes (Lippa et al. 1979), [^3H]flunitrazepam binding in cortical and cerebellar membranes from LS and SS mice was assayed in the presence of this compound at both 0 and 37 °C. When comparing the potency of CL 218,872 to inhibit radioligand binding to benzodiazepine receptors in these lines, we could detect no differences for either 50 percent inhibitory concentrations

Table 2.—CL 218,872 inhibition of [3 H]flunitrazepam binding in LS and SS cortical and cerebellar membranes incubated at 0 and 37 °C^a

Line	0 °C				37 °C			
	Cortex		Cerebellum		Cortex		Cerebellum	
	IC ₅₀ (μ M)	n_H	IC ₅₀ (μ M)	n_H	IC ₅₀ (μ M)	n_H	IC ₅₀ (μ M)	n_H
LS	0.353 \pm 0.11	0.56 \pm 0.1	0.115 \pm 0.02	0.83 \pm 0.1	0.885 \pm 0.22	0.75 \pm 0.1	0.566 \pm 0.19	0.89 \pm 0.1
SS	0.396 \pm 0.09	0.59 \pm 0.1	0.113 \pm 0.02	0.84 \pm 0.1	0.962 \pm 0.57	0.67 \pm 0.1	0.595 \pm 0.07	0.83 \pm 0.1

³H]flunitrazepam binding to well-washed (50 mM Tris-citrate, pH 7.4) membranes was determined by using \approx 1.0 nM radioligand at 0 °C and \approx 5.0 nM at 37 °C as well as 25 nM–12.8 μ M CL 218,872. Values represent the mean \pm SEM of at least three mice, calculated by using GRAPHPAD (ISI). n_H is the Hill coefficient. In separate experiments using diazepam (0.39–100 nM at 0 °C; 1.56–400 nM at 37 °C) to displace [3 H]flunitrazepam in cerebellar membranes washed and incubated in 50 mM Tris-HCl (pH 7.4), cerebellar Hill coefficients approached unity.

(IC₅₀s) or Hill coefficients in either region (table 2). As previously reported, however, cortical Hill coefficients from both lines were less than unity, and cerebellar IC₅₀s were significantly lower than corresponding cortical values in both lines.

CL 218,872 has been reported to be an efficacious anxiolytic and anticonvulsant with little or no hypnotic potential (Lippa et al. 1982; Gee et al. 1983). Nevertheless, when administered to these selected lines at doses higher than those needed to produce its anticonflict effects, a differential pattern of hypnosis was observed (data not shown). The LS mice were noticeably impaired at 60 mg/kg while none of the SS mice lost the righting reflex at this dose. At higher doses (90-120 mg/kg), the SS mice were also impaired, and the differences between the two lines not as marked. These findings suggest that although the proportions of type I to type II benzodiazepine receptors are similar, the lines may still exhibit selectivity to the hypnotic activity of C1 218,872, as they do to other benzodiazepine receptor ligands (McIntyre and Alpern, 1985; Marley et al. 1986). Alternatively, these findings may also indicate that although the recognition site qualities of the type I and type II benzodiazepine receptors are similar in the LS and SS lines, the manner in which either or both subtypes are coupled to the GABA receptor may differ between the lines.

That differences in the coupling of the individual components of the supramolecular complex may explain some of the findings reported thus far is further supported by experiments using the "cage" convulsant [³⁵S]t-butylbicyclophosphorothionate (TBPS). Electrophysiologic and biochemical evidence (Squires et al. 1983; Akaike et al. 1985; Havoundjian et al. 1986; Van Renterghem et al. 1987) suggests that cage convulsants such as TBPS or picrotoxin act inside GABA-gated chloride channels, which may be formed by the membrane-spanning domains of the benzodiazepine and GABA receptors (Schofield et al. 1987). Scatchard analysis using [³⁵S]TBPS (24 °C) revealed a significantly lower K_d for membranes from SS than from LS mice but no differences for B_{max} (table 3).

Table 3.— K_d and B_{\max} values for [35 S]TBPS binding to LS and SS cortical membranes^a

Line	K_d (nM)	B_{\max} (fmol/mg of protein)
LS	47.9 ± 2^b	$1,347 \pm 54$
SS	38.8 ± 1	$1,413 \pm 99$

^aAssays were performed on one animal per cage each day as described previously (McIntyre et al. 1988). Values were estimated from Scatchard plots (correlation coefficients > 0.985) and represent the mean \pm SEM for 16 animals per group.

^bSignificantly different from corresponding SS value, Student's *t*-test, $P < 0.001$.

Conclusions

In summary, behavioral, pharmacologic, and biophysical evidence implicates the benzodiazepine-GABA receptor-chloride ionophore complex in the differential response of the LS and SS mouse lines to depressants and convulsants. Thus, general anesthetics, barbiturates, benzodiazepines, and alcohols have a much more profound effect on LS than SS mice, whereas many convulsants precipitate clonus more readily in SS than LS mice (McIntyre and Alpern 1985, 1986b; Marley et al. 1986). Since an accumulating body of evidence suggests that the supramolecular complex mediates the effects of these agents (Skolnick and Paul 1988), it seems reasonable to conclude that at least one of the loci bidirectionally selected for in this instance modulates the supramolecular complex. This conclusion does not imply that all of the loci which have been differentially selected in these lines code for components of the supramolecular complex. Nevertheless, no other neural effector system seems to be able to account for so many of the differences cited above.

Whether these findings can be extrapolated to a more comprehensive hypothesis concerning the initial effects of ethanol remains to be seen. We

have suggested that a number of phenotypic responses which are either positively or negatively correlated with the originally selected trait are not related fortuitously. Indeed, the results reported here suggest that derivation of the LS and SS lines may have actually selected for aspects of an effector system that mediates not only ethanol-induced hypnosis but pharmacologically related traits as well. Furthermore, since preliminary results from other selection studies involving ethanol sensitivity (in rats) and benzodiazepine sensitivity indicate that similar mechanisms may have been bidirectionally selected (Hellevuo et al. 1987; Allan et al. 1988), this may be a common result of selecting for depressant sensitivity.

Although it cannot be ignored that these lines are somewhat inbred (McClearn and Kakihana 1981), other explanations for these correlated responses are just as plausible. Usually, in order to partition legitimately correlated phenotypic responses from those arising spuriously, one would examine replicate selected "high" and "low" lines to determine whether these correlations occurred to the same degree in both sets of replicates over several generations (Crabbe et al. 1987). Unfortunately, replicate selected lines were not developed in this instance, limiting somewhat the theoretical value of these selected lines (Deitrich and Spuhler 1984; Crabbe and Kosubud 1986). Nevertheless, as the results reported here suggest, the LS and SS selected lines remain a powerful pharmacogenetic model for investigating the etiology of alcoholism.

References

- Akaike, N.; Hattori, K.; Oomura, Y.; and Carpenter, D. Bicuculline and picrotoxin block γ -aminobutyric acid-gated Cl^- conductance by different mechanisms. *Experientia* 41:70-71, 1985.
- Allan, A.M.; Gallaher, E.J.; Gionet, S.E.; and Harris, R.A. Genetic selection for benzodiazepine ataxia produces functional changes in the γ -aminobutyric acid receptor chloride channel complex. *Brain Res* 452:118-126, 1988.

- Allan, A.M., and Harris, R.A. Gamma-aminobutyric acid and alcohol actions: Neurochemical studies in long-sleep and short-sleep mice. *Life Sci* 39:2005-2015, 1986.
- Alpern, H.P., and McIntyre, T.D. Evidence that the selectively bred long- and short-sleep mouse lines display common narcotic reactions to many depressants. *Psychopharmacology* 85:456-459, 1985.
- Alpern, H.P., and McIntyre, T.D. Sedative-hypnotic anomalies related to dose of pentobarbital in long-sleep and short-sleep selectively-bred mice. *Pharmacol Biochem Behav* 25:333-336, 1986.
- Crabbe, J.C., and Kosobud, A. Ethanol withdrawal in mice bred to be genetically prone or resistant to ethanol withdrawal seizures. *J Pharmacol Exp Ther* 238:170-177, 1986.
- Crabbe, J.C.; Kosubud, A.; and Tam, B.R. Genetic selection of mouse lines sensitive (COLD) and resistant (HOT) to acute ethanol hypothermia. *Alcohol Drug Res* 7:163-174, 1987.
- Crabbe, J.C.; Kosobud, A.; Young, E.R.; Tam, B.R.; and McSwigan, J.D. Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus*. *Behav Gen* 15:521-536, 1985.
- Deitrich, R.A., and Spuhler, K. Genetics of alcoholism and alcohol actions. In: Smart, R.G.; Cappell, H.C.; Glaser, F.B.; Israel, Y.; Kalant, H.; Popham, R.E.; Schmidt, W.; and Sellars, E.M., eds. *Research Advances in Alcohol and Drug Problems*. New York: Plenum Press, 1984. pp. 47-98.
- Dudek, B.C., and Phillips, T.J. Locomotor stimulant and intoxicant properties of methanol, ethanol, tertiary butanol, and pentobarbital in long-sleep and short-sleep mice. *Subst Alcohol Actions Misuse* 4:31-36, 1983.
- Dunwiddie, T.V., and Proctor, W.R. Behavioral sensitivity to purinergic drugs parallels ethanol sensitivity in selectively bred mice. *Science* 217:519-521, 1984.

- Eriksson, K. Genetic selection for voluntary alcohol consumption in the albino rat. *Science* 159:739-741, 1968.
- Eriksson, K., and Rusi, M. Finnish selection studies on alcohol-related behaviors: General outline. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. N. ADM(81-1113). Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981, pp. 87-117.
- Erwin, V.E.; Heston, W.D.W.; McClearn, G.E.; and Deitrich, R.A. Effects of hypnotics on mice genetically selected for sensitivity to ethanol. *Pharmacol Biochem Behav* 4:679-683, 1976.
- Gavish, M., and Snyder, S.H. Benzodiazepine-recognition sites on GABA receptors. *Nature* (London) 287:651-652, 1980.
- Gee, K.W.; Brinton, R.E.; and Yamamura, H.I. CL 218872 antagonism of diazepam induced loss of righting reflex: Evidence for partial agonistic activity at the benzodiazepine receptor. *Life Sci* 32:1037-1040, 1983.
- Goeders, N.E.; Ritz, M.C.; and Kuhar, M.J. Buspirone enhances benzodiazepine receptor binding in vivo. *Neuropharmacology* 27:275-280, 1988.
- Goldstein, D.B.; Chin, J.H.; and Lyon, R.C. Ethanol disordering of spin-labeled mouse brain membranes: Correlation with genetically determined ethanol sensitivity of mice. *Proc Natl Acad Sci USA* 79:4231-4233, 1982.
- Greer, C.A., and Alpern, H.P. Mediation of myoclonic seizures by dopamine and clonic seizures by acetylcholine and GABA. *Life Sci* 21:385-392, 1977.
- Harris, R.A.; Zaccaro, L.M.; McQuilken, S.; and McClard, A. Effects of ethanol and calcium on lipid order of membranes from mice selected for genetic differences in ethanol intoxication. *Alcohol* 5:251-257, 1988.
- Havoundjian, H.; Paul, S.; and Skolnick, P. The permeability of γ -aminobutyric acid-gated chloride channels is described by the binding of a cage convulsant, [35 S]t-butylbicyclophosphorothionate. *Proc Natl Acad Sci USA* 83:9241-9244, 1986.

- Hellevuo, K.; Kiianmaa, K.; Juhakoski, A.; and Kim, C. Intoxicating effects of lorazepam and barbital in rat lines selected for differential sensitivity to ethanol. *Psychopharmacology* 91:263-267, 1987.
- Heston, W.D.W.; Erwin, V.E.; Anderson, S.M.; and Robbins, H. A comparison of the effects of alcohol on mice selectively bred for differences in ethanol sleep-time. *Life Sci* 14:365-370, 1974.
- Kakihana, R.; Brown, D.R.; McClearn, G.E.; and Tabershaw, I.R. Brain sensitivity to alcohol in inbred mouse strains. *Science* 154:1574-1575, 1966.
- Klepner, C.A.; Lippa, A.S.; Benson, D.I.; Sano, M.C.; and Beer, B. Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors. *Pharmacol Biochem Behav* 11:457-462, 1979.
- Koblin, D.D., and Deady, J.E. Anesthetic requirement in mice selectively bred for difference in ethanol sensitivity. *Br J Anaesthesiol* 53:5-10, 1981.
- Li, T.-K., and Lumeng, L. Alcohol metabolism of inbred strains of rats with alcohol preference and nonpreference. In: Thurman, R.G.; Williamson, J.R.; Drott, H.; and Chance, B., eds. *Alcohol and Aldehyde Metabolizing Systems*. Vol. 3. New York: Academic Press, 1977. pp. 625-633.
- Lippa, A.S.; Coupet, J.; Greenblatt, E.N.; Klepner, C.A.; and Beer, B. A synthetic non-benzodiazepine ligand for benzodiazepine receptors: A probe for investigating neuronal substrates of anxiety. *Pharmacol Biochem Behav* 11:99-106, 1979.
- Lippa, A.S.; Meyerson, R.L.; and Beer, B. Molecular substrates of anxiety: Clues from the heterogeneity of benzodiazepine receptors. *Life Sci* 31:1409-1417, 1982.
- Malila, A. Intoxicating effects of three aliphatic alcohols and barbital on two rat strains genetically selected for their ethanol intake. *Pharmacol Biochem Behav* 8:197-201, 1978.
- Marley, R.J.; Miner, L.L.; Wehner, J.M.; and Collins, A.C. Differential effects of central nervous system depressants in long-sleep and short-sleep mice. *J Pharmacol Exp Ther* 238:1028-1033, 1986.

- Marley, R.J., and Wehner, J.M. GABA enhancement of flunitrazepam binding in mice selectively bred for differential sensitivity to ethanol. *Alcohol Drug Res* 7:25-32, 1987.
- Martz, A.; Deitrich, R.A.; and Harris, R.A. Behavioral evidence for the involvement for γ -aminobutyric acid in the actions of ethanol. *Eur J Pharmacol* 89:53-62, 1983.
- McClearn, G.E. Animal models of genetic factors in alcoholism. In: Mello, N.K., ed. *Advances in Substance Abuse*. Vol. 11. Greenwich, CT: Jai Press, 1981. pp. 25-51.
- McClearn, G.E., and Kakihana, R. Selective breeding for ethanol sensitivity in mice. *Behav Genet* 3:409-410, 1973.
- McClearn, G.E., and Kakihana, R. Selective breeding for ethanol sensitivity: Short-sleep and long-sleep mice. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds. *Development of Animal Models as Pharmacogenetic Tools*. DHHS Pub. No. 81-1113. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 147-159.
- McIntyre, T.D., and Alpern, H.P. The interaction of CNS depressants of GABAergic drugs in mice selectively-bred for the narcotic effects of ethanol. *Soc Neurosci Abstr* 9:130, 1983.
- McIntyre, T.D., and Alpern, H.P. Reinterpretation of the literature indicates differential sensitivities of long-sleep and short-sleep mouse lines are not specific to alcohol. *Psychopharmacology* 87:379-389, 1985.
- McIntyre, T.D., and Alpern, H.P. Thiopental, phenobarbital, and chlordiazepoxide induce the same differences in narcotic reaction as ethanol in long-sleep and short-sleep selectively-bred mice. *Pharmac Biochem Behav* 24:895-898, 1986a.
- McIntyre, T.D., and Alpern, H.P. Further evidence of a GABA mechanism distinguishing long sleep and short sleep mice. *Soc Neurosci Abstr* 12:921, 1986b.

- McIntyre, T.D.; Trullas, R.; and Skolnick, P. Differences in biophysical properties of the benzodiazepine/GABA receptor-chloride channel complex in the long-sleep and short-sleep mouse lines. *J Neurochem* 51:642-647, 1988.
- Miller, L.G.; Greenblatt, D.J.; Barnhill, J.G.; and Shader, R.I. Differential modulation of benzodiazepine receptor binding by ethanol in LS and SS mice. *Pharmacol Biochem Behav* 29:471-477, 1988.
- Mohler, H., and Okada, T. Benzodiazepine receptor: Demonstration in the central nervous system. *Science* 198:849-851, 1977.
- Palmer, M.R.; Olson, L.; Dunwiddie, T.V.; Hoffer, B.J.; and Seiger, A. Neonatal cerebellectomy alters ethanol-induced sleep time of short-sleep but not long-sleep mice. *Pharmacol Biochem Behav* 20:153-159, 1984.
- Palmer, M.R.; Wang, Y.; Fossum, L.H.; and Spuhler, K. Genetic correlation of ethanol-induced ataxia and cerebellar Purkinje neuron depression in inbred rat strains and selected lines of rats. *Alcoholism Clin Exp Res* 10:494-501, 1987.
- Phillips, T.J., and Dudek, B.C. Bicuculline-induced seizures in mice which differ in sensitivity to ethanol. *Soc Neurosci Abstr* 9:1240, 1983.
- Riley, E.P.; Freed, E.X.; and Lester, D. Selective breeding of rats for differences in reactivity to alcohol. An approach to an animal model of alcoholism. I. General procedure. *Q J Studies Alcohol* 37:1535-1547, 1976.
- Riley, E.P.; Lochry, E.A.; and Freed, E.X. Differential tolerance to pentobarbital in rats bred for differences in alcohol sensitivity. *Psychopharmacology* 58:167-170, 1978.
- Riley, E.P.; Shapiro, N.R.; and Lochry, E.A. Hypnotic susceptibility to various depressants in rats selected for differential ethanol sensitivity. *Psychopharmacology* 60:311-312, 1979.
- Roberts, R.C. Current perspectives on selective breeding: Theoretical aspects. In: McClearn, G.E.; Deitrich, R.A.; and Erwin, V.G., eds.

Development of Animal Models as Pharmacogenetic Tools. DHHS Pub. No. (ADM)81-1113. Washington, DC: Supt. of Docs., U.S. Govt. Print. Off., 1981. pp. 37-58.

Schofield, P.R.; Darlison, M.G.; Fujita, N.; Burt, D.R.; Stephenson, F.A.; Rodriguez, H.; Rhee, L.M.; Ramachandran, J.; Reale, V.; Glencorse, T.A.; Seeburg, P.H.; and Barnard, E. Sequence and functional expression of the GABA_A receptor shows a ligand-gated receptor super-family. *Nature* (London) 328:221-227, 1987.

Sieghart, W., and Karobath, M. Molecular heterogeneity of benzodiazepine receptors. *Nature* (London) 286:285-287, 1980.

Skolnick, P., and Paul, S. The benzodiazepine/GABA receptor chloride channel complex. *ISI Atlas Pharmacol* 2:19-22, 1988.

Sorensen, S.; Dunwiddie, T.V.; McClearn, G.; Freedman, R.; and Hoffer, B.J. Ethanol-induced depressions in cerebellar and hippocampal neurons of mice selectively bred for differences in ethanol sensitivity: An electrophysiological study. *Pharmacol Biochem Behav* 14:227-234, 1981.

Sorensen, S.; Palmer, M.R.; Dunwiddie, T.V.; and Hoffer, B.J. Electrophysiological correlates of ethanol-induced sedation in differentially sensitive lines of mice. *Science* 210:1143-1145, 1980.

Speth, R.C.; Wastek, G.J.; Johnson, P.C.; and Yamamura, H.I. Benzodiazepine binding in human brain: Characterization using 3H-flunitrazepam. *Life Sci* 22:859-866, 1978.

Squires, R., ed. *GABA and Benzodiazepine Receptors*. Boca Raton: CRC Press, 1988.

Squires, R.F.; Benson, D.I.; Braestrup, C.; Coupet, J.; Klepner, C.A.; Myers, V.; and Beer, B. Some properties of brain specific benzodiazepine receptors: New evidence for multiple receptors. *Pharmacol Biochem Behav* 10:825-830, 1979.

- Squires, R., and Braestrup, C. Benzodiazepine receptors in rat brain. *Nature* (London) 266:732-734, 1977.
- Squires, R.; Casida, J.; Richardson, M.; and Saederup, E. [³⁵S]t-butylbicyclophosphorothionate binds with high affinity to brain specific sites coupled to γ -aminobutyric acid-A and recognition sites. *Mol Pharmacol* 23:326-336, 1983.
- Sudzak, P.D.; Glowa, J.R.; Crawley, J.N.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. A selective imidazobenzodiazepine antagonist of ethanol in the rat. *Science* 234:1243-1247, 1986.
- Tallman, J.; Paul, S.; Skolnick, P.; and Gallagher, D. Receptors for the age of anxiety: The pharmacology of benzodiazepines. *Science* 207:274-281, 1980.
- Trullas, R.; Havoundjian, H.; and Skolnick, P. Stress-induced changes in [³⁵S]t-butylbicyclophosphorothionate binding to γ -aminobutyric acid-gated chloride channels are mimicked by in vitro occupation of benzodiazepine receptors. *J Neurochem* 49:968-974, 1987.
- Van Renterghem, C.; Bilbe, G.; Moss, S.; Smart, T.; Constanti, A.; Brown, D.; and Barnard, E. GABA receptors induced in *Xenopus* oocytes by chick brain mRNA: Evaluation of TBPS as a use-dependent channel blocker. *Mol Brain Res* 2:21-31, 1987.
- Wilson, J.R.; Erwin, V.G.; DeFries, J.C.; Peterson, D.R.; and Cole-Harding, S. Ethanol dependence in mice: Direct and correlated responses to ten generations of selective breeding. *Behav Gen* 14:235-256, 1984.

Ethanol and GABAergic Transmission¹

*Maharaj K. Ticku, A. K. Mehta, M. Mhatre,
and S. K. Kulkarni²*

Introduction

γ -Aminobutyric acid_A (GABA_Aergic) synapse is a site of action for drugs possessing antianxiety, hypnosedative, anticonvulsant, and anesthetic properties (Olsen 1982; Ticku 1983; Rastogi and Ticku 1986). Ethanol was found to have several pharmacologic actions akin to those of benzodiazepines and barbiturates that facilitated GABA_Aergic transmission (Ticku 1983; Rastogi and Ticku 1986). Ethanol is reported to influence the GABA-benzodiazepine receptor-ionophore complex (Hunt 1983; Liljequist and Engel 1982; Ticku and Kulkarni 1988). It inhibited the *in vitro* binding of [³⁵S]*t*-butylbicyclophosphorothionate to brain membranes (Thyagarajan and Ticku 1985; Liljequist et al. 1986; Rastogi et al. 1986) and facilitated GABA-gated Cl⁻ conductance in cultured neurons (Ticku et al. 1986; Mehta and Ticku 1988), microsacs (Allan and Harris 1986), and synaptoneuroosomes (Suzdak et al. 1986a). More recent *in vivo* and *in vitro* studies have shown that the imidazobenzodiazepine Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-Oxo-4H-imidazo [1,5 α] [1,4]-benzodiazepine-3-carboxylate) antagonizes the behavioral and biochemical effects of ethanol (Suzdak et al. 1986a; Bonetti et al. 1985; Lister 1987). The present study was aimed at

¹This work was supported by grant AA-04090 from NIAAA.

²Department of Pharmacology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284-7764.

investigating the interaction between ethanol and the partial inverse agonist Ro 15-4513, using three different parameters, behavioral, biochemical (binding), and functional ($^{36}\text{Cl}^-$ influx), to resolve the specificity of Ro 15-4513 as an ethanol antagonist and to explore the molecular mechanism underlying the acute and chronic effects of ethanol.

Methods

Animals

Adult male Sprague-Dawley rats (200-250 g) maintained on a 12-hr dark/light cycle were used for behavioral and biochemical studies. The animals had free access to food and water except during experimentation. Female and male C57BL/6J mice (8-10 weeks old) from Jackson Laboratories were used for neuronal cell culture experiments. Spinal cords were dissected from 13- to 14-day old mouse embryos, and spinal neurons were grown on sterile coverslips as described previously (Mehta and Ticku 1988).

Behavioral Parameters

REVERSAL OF THE ANTICONVULSANT ACTION OF ETHANOL BY RO 15-4513

Ethanol (2 g/kg) is reported to possess anticonvulsant action against bicuculline (8 mg/kg intraperitoneally [IP]) and picrotoxin (10 mg/kg). The reversal by Ro 15-4513 (1 and 4 mg/kg IP) of the anticonvulsant action of ethanol as well as that of pentobarbital was studied. Furthermore, these effects of Ro 15-4513 were compared with those of FG 7142, another partial inverse agonist of benzodiazepine receptors.

EFFECT OF CHRONIC ETHANOL TREATMENT ON THE PROCONVULSANT ACTION OF RO 15-4513

The proconvulsant effect of Ro 15-4513 against bicuculline- and picrotoxin-induced convulsions in rats was investigated. The proconvulsant/convulsant effect of Ro 15-4513 and/or pentylenetetrazol (PTZ) in rats chronically treated with ethanol and during ethanol withdrawal was studied to explore

the possibility of alteration in the receptor sensitivity after chronic ethanol treatment.

Biochemical Studies

The effects of chronic ethanol treatment on the binding characteristics of [³H]Ro 15-4513 in rat brain cerebral cortex, cerebellum, hippocampus, and striatum were investigated. Rats were chronically treated with ethanol (5 g/kg, 20 percent [wt/vol], three times a day for 6 days) by the intragastric intubation method (Mhatre et al. 1988). Rats were decapitated at the end of the treatment (control and chronically treated rats) or 24 hr after chronic treatment (ethanol-withdrawn rats), and various brain regions were dissected. The mitochondrial-plus-microsomal ($P_2 + P_3$) membrane preparation was made as described earlier (Rastogi et al. 1986). Aliquots of the membrane fractions were incubated with [³H]Ro 15-4513 (0.25-20 nM) for 60 min at 0-4 °C. Binding was determined by the filtration method (Mhatre et al. 1988). Nonspecific binding was determined in the presence of 2×10^{-6} M nonradioactive Ro 15-4513 and was subtracted from total binding. The K_d and B_{max} values were obtained by the linear regression of Scatchard plot data.

Functional Assay

³⁶Cl⁻ influx was measured by a modification of the method of Thampy and Barnes (1984) as described by Mehta and Ticku (1988). Briefly, coverslips with attached neuronal cells (7-8 days old) were removed from tissue culture medium and rinsed twice at room temperature in HEPES-buffered saline (pH 7.4) for 3-4 s. Coverslips were drained and transferred to 2 ml of buffer (22 °C) containing ³⁶Cl (2 μCi/ml) in the absence or presence of various drugs. The influx was terminated after 10 s by rapid transfer of the coverslip to another beaker containing 1,000 ml of ice-cold stop solution. The coverslips were drained and transferred to scintillation vials containing 0.2 N NaOH. After removal of a 0.5-ml aliquot for protein estimation, the contents were neutralized (1 N HCl) and 10 ml of hydrofluor was added to the vials; radioactivity was determined by liquid scintillation spectrometry. Protein estimation was done by bicinchorinic (BCA) protein assay.

Materials

Ro 15-4513 was a gift from Dr. W. Haefely (Hoffmann-La Roche, Basel). [³H]Ro 15-4513 and ³⁶Cl (HCl) were purchased from New England Nuclear (Boston, MA) and ICN Radiochemicals (Irvine, CA), respectively. Other drugs used were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Statistical analysis of the data was done by one-way analysis of variance with 95 percent confidence ($P < 0.05$) and by comparison with Student's *t* test. Fisher's exact test was used to assess the significant difference in mortality (percent) between various groups.

Results

Behavioral Parameters

REVERSAL OF THE ANTICONVULSANT EFFECT OF ETHANOL

Ethanol (2 g/kg IP) offered complete protection against the tonic convulsive phase of bicuculline- and picrotoxin-induced convulsions. It offered 33 and 100 percent protection against mortality due to bicuculline- and picrotoxin-induced mortality, respectively (Kulkarni and Ticku 1989). Pretreatment with Ro 15-4513 (4 mg/kg) reversed the protective effect of ethanol (33 versus 13 percent; 100 versus 50 percent; figure 1). The reversal of the Ro 15-4513 effect was sensitive to blockade by Ro 15-1788 (10 mg/kg), a benzodiazepine receptor antagonist.

Ro 15-4513 partially reversed the protective effect of pentobarbital against bicuculline- or picrotoxin-induced convulsions (Kulkarni and Ticku in press) (figure 2). However, FG 7142 was not effective in doing so.

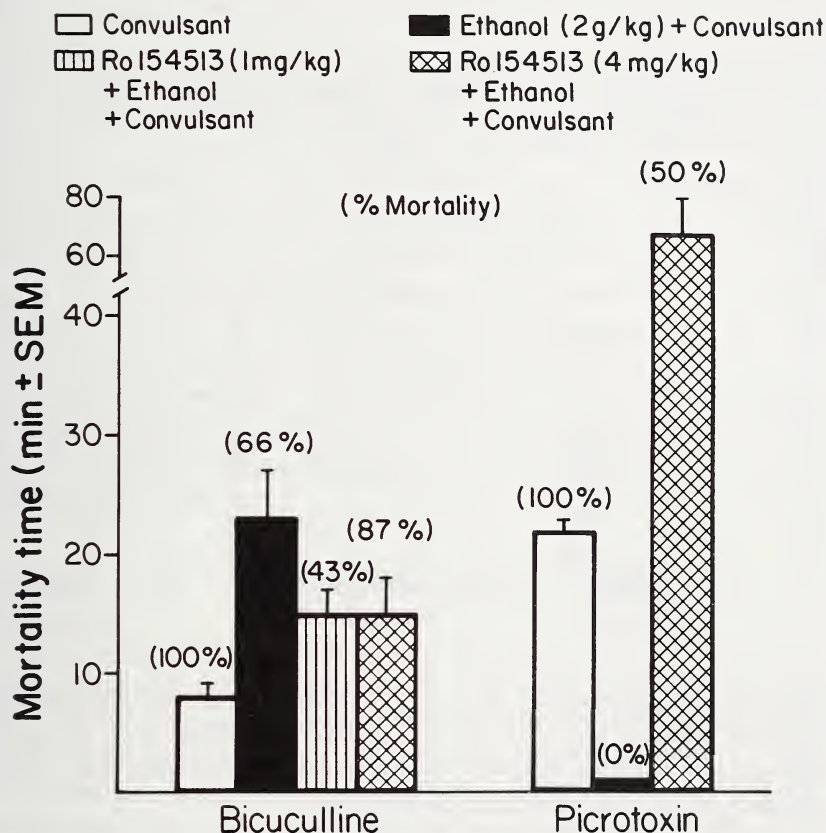


Figure 1.—Modification by Ro 15-4513 (1 and 4 mg/kg) of the anticonvulsant effect of ethanol against bicuculline- and picrotoxin-induced convulsions as assessed by delay in mortality time and percent protection against mortality in rats.

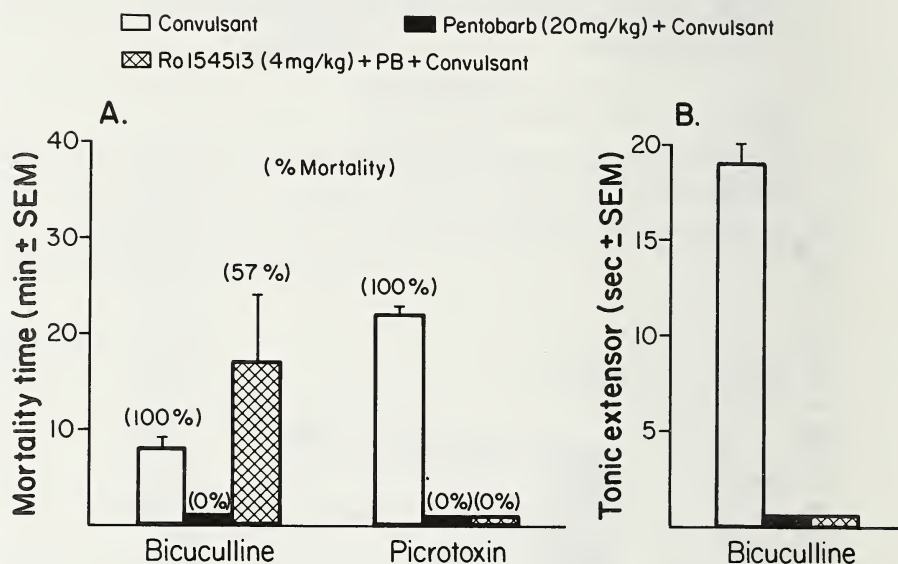


Figure 2.—Modification by Ro 15-4513 (4 mg/kg) of the anticonvulsant effect of pentobarbital (20 mg/kg) against bicuculline- and picrotoxin-induced convulsions as assessed by delay in onset of mortality time, percent mortality, and protection of tonic phase in rats.

EFFECT OF CHRONIC ETHANOL TREATMENT ON THE PROCONVULSANT ACTION OF Ro 15-4513

Both Ro 15-4513 and FG 7142 had a proconvulsant effect against bicuculline but not against picrotoxin. In the chronic ethanol-maintained rats, the threshold to PTZ-induced convulsions was increased from 80 mg/kg (control) to 200 mg/kg (Mehta and Ticku in press). Conversely, in the ethanol-withdrawal group, the threshold was decreased from 80 mg/kg (control) to 40 mg/kg. Ro 15-4513, which did not show any effect per se in control rats, produced clonic-tonic convulsions, susceptible to blockade by diazepam or Ro 15-1788, in the ethanol-withdrawal group. On the other hand, FG 7142 had no such effect. However, Ro 15-4513 as well as FG 7142 produced a proconvulsant effect against PTZ in control and ethanol withdrawal groups. Interestingly, FG 7142, unlike Ro 15-4513, did not elicit a proconvulsant effect in chronic ethanol-maintained rats (table 1; Mehta and Ticku in press).

Biochemical Studies

[³H]Ro 15-4513 was found to bind in a specific and saturable manner to discrete regions of the rat brain (Mhatre et al. 1988). The binding was also displaced in a concentration-dependent manner by Ro 15-4513, Ro 15-1788, and diazepam. Scatchard plots of the binding of [³H]Ro 15-4513 in cortex and cerebellum revealed that specific [³H]Ro 15-4513 binding was increased selectively after chronic ethanol treatment in cerebral cortex (61 percent) and cerebellum (77 percent) but not in hippocampus and striatum (Mhatre et al. 1988). The increase was due to an increase in the B_{\max} value in both regions, without any significant effect in the K_d value. At 24 hr after withdrawal, the B_{\max} values returned to normal in cortex but not in cerebellum (Mhatre et al. 1988; table 2). In contrast to [³H]Ro 15-4513, specific binding of [³H]flunitrazepam or [³H]Ro 15-1788 was not altered after chronic ethanol treatment in any of the brain regions tested (table 3). Similar results were obtained when spinal cultured neurons were exposed to 50 mM ethanol for 3 days (M. Mhatre and M. K. Ticku, unpublished observations).

Table 1.—Modification of PTZ-induced convulsions in control, alcohol-maintained, and alcohol withdrawal groups of rats by Ro 15-4513 and FG 7142^a

Treatment (mg/kg)	Observed response in given group		
	Control	Alcohol maintained	Alcohol withdrawal
PTZ (40)	Partial convulsant	Nonconvulsant	Full convulsant Blocked by diazepam but not by Ro 15-1788
PTZ (80)	Full convulsant Blocked by diazepam but not by Ro 15-1788	Nonconvulsant	Full convulsant Blocked by diazepam but not by Ro 15-1788
Ro 15-4513 (5) (10)	Nonconvulsant	Nonconvulsant	Convulsant Blocked by diazepam and by Ro 15-1788
Ro 15-4513 (2) + PTZ (20/40)	Proconvulsant (PTZ [40]) Blocked by diazepam or Ro 15-1788	Nonproconvulsant	Proconvulsant (PTZ [20]) Blocked by diazepam or Ro 15-1788
Ro 15-4513 (4) + PTZ (160)	—	Proconvulsant Blocked by diazepam and by Ro 15-1788	—
FG 7142 (20) + PTZ (20/40)	Proconvulsant (PTZ [40]) Blocked by diazepam or Ro 15-1788	Nonproconvulsant	Proconvulsant (PTZ [20]) Blocked by diazepam or Ro 15-1788

^aRats were chronically (20 percent [wt/vol] three times a day for 6 days) treated with alcohol. In the alcohol-maintained and withdrawal groups, experiments were performed 1 and 24 hr, respectively, after the last dose of ethanol.

Table 2.—Effect of chronic ethanol administration on the binding pattern of [³H]Ro 15-4513 in different regions of rat brain (Scatchard plot analysis)

Brain region	Specific binding of [³ H]Ro 15-4513 ^a			
	Chronic		Withdrawn	
	K_d (nM)	B_{max} (pmol/mg of protein)	K_d (nM)	B_{max} (pmol/mg of protein)
Cerebral cortex	0.61±0.09 (0.72±0.40)	3.404±0.43 ^b (2.116±0.40)	0.83±0.34	2.557±0.27
Cerebellum	0.84±0.25 (0.57±0.18)	2.576±0.33 ^b (1.452±0.22)	0.70±0.27	2.446±0.23 ^b

^aExpressed as the mean ± standard deviation of at least four to six experiments.

^b $P < 0.001$ as compared with the control values given in parentheses. No significant change in binding pattern was observed in hippocampus and striatum. (Modified from Mhatre et al. 1988.)

Functional Assay

Cultured spinal cord neurons contain benzodiazepine receptor sites, which are coupled to GABA, picrotoxin, and barbiturate sites (Mehta and Ticku 1987). These cells also respond to GABA agonists by an increase in ³⁶Cl⁻ influx, which has the specificity of the GABA_A receptor system (Mehta and Ticku 1988). At concentrations of ≥50 mM, ethanol increased ³⁶Cl⁻ influx per se (table 4). This direct effect was sensitive to blockade by bicuculline or picrotoxin, indicating the involvement of GABA_A receptor-coupled Cl⁻ channels in this action (Mehta and Ticku 1988). The direct effect of ethanol was also blocked by the benzodiazepine receptor inverse agonists Ro 15-4513 and FG 7142, Ro 15-4513 being the more potent. Ethanol (5-100 mM) also produced a concentration-dependent enhancement of GABA (10 μM)-

Table 3.—Effect of chronic ethanol treatment on binding of ligands to the benzodiazepine receptor

Brain region	Specific binding (fmol/mg of protein) ^a		
	Control	Chronic	Withdrawal
^[3H] Ro 15-4513			
Cerebral cortex	1,018 ± 131 (8) ^b	2,019 ± 109 (3) ^c	1,212 ± 248 (6)
Cerebellum	942 ± 133 (5)	1,317 ± 195 (6) ^d	1,480 ± 207 (5) ^c
^[3H] Ro 15-1788			
Cerebral cortex	742 ± 57 (4)	804 ± 21 (4)	773 ± 58 (4)
Cerebellum	254 ± 10 (3)	260 ± 18 (4)	285 ± 18 (4)
^[3H] flunitrazepam			
Cerebral cortex	634 ± 30 (4)	718 ± 11 (4)	675 ± 20 (4)
Cerebellum	360 ± 40 (4)	358 ± 30 (4)	369 ± 40 (4)

^aMeasured as described by Mhatre et al. (1988).^bNumbers in parentheses indicate number of experiments^c*P* < 0.001 as compared with control.^d*P* < 0.002 as compared with control.

induced ³⁶Cl⁻ influx (figure 3). The concentration of ethanol that produced half-maximal enhancement (EC₅₀) was 15 mM. This enhancing effect of ethanol was sensitive to blockade by bicuculline or picrotoxin. Both Ro 15-4513 and FG 7142 also produced a concentration-dependent decrease in GABA-stimulated ³⁶Cl⁻ influx as well as an ethanol-enhancing effect on GABA-induced ³⁶Cl⁻ influx (Mehta and Ticku 1988). These effects of Ro 15-4513 were sensitive to blockade by Ro 15-1788, a benzodiazepine receptor antagonist.

Table 4.—Direct effect of ethanol on $^{36}\text{Cl}^-$ influx in cultured spinal cord neurons^a

Ethanol (mM)	% Increase over basal
20 (9)	2.5 ± 1.9
50 (9)	34.3 ± 3.9 ^b
100 (3)	37.1 ± 6.9 ^b
500 (3)	38.5 ± 3.1 ^b

^aCoverslips with attached cells were washed twice in physiologic buffer before incubation with ethanol and ^{36}Cl for 10s as described in the text. Values represent the mean ± standard deviation for the number of experiments indicated in parentheses.

^b $P < 0.001$ as compared with 20 mM ethanol. (From Mehta and Ticku 1988.)

Discussion

Recent experimental evidence suggests that some of the neuropharmacologic and behavioral actions of ethanol may be mediated through the central inhibitory synaptic transmitter GABA (Cott et al. 1976; Hunt 1983; Ticku 1983; Liljequist and Engel 1982; Rastogi and Ticku 1986). Ethanol potentiated the inhibition of cortical neurons by GABA without affecting the inhibition caused by other putative neurotransmitter systems (Nestoros 1980). Since ethanol shared several of its behavioral effects with barbiturates and benzodiazepines, it has been speculated the GABA-benzodiazepine receptor-coupled chloride channels could be the site of action of ethanol in the brain (Ticku 1983; Mendelson et al. 1985; Rastogi and Ticku 1985, 1986; Liljequist et al. 1986; Rastogi et al. 1986). More direct in vitro evidence using synaptoneurosome indicated that at pharmacologically relevant concentrations, ethanol potentiated muscimol stimulation of $^{36}\text{Cl}^-$ uptake (Harris and Allan 1985; Suzdak et al. 1986b), and these effects were sensitive to blockade by both bicuculline and picrotoxin (Suzdak et al. 1986b). Our laboratory has demonstrated that in intact cultured spinal cord neurons, ethanol (5-100 mM) potentiated GABA-mediated $^{36}\text{Cl}^-$ influx; at higher concentrations (≥ 50 mM), it directly activated Cl channels in these neurons.

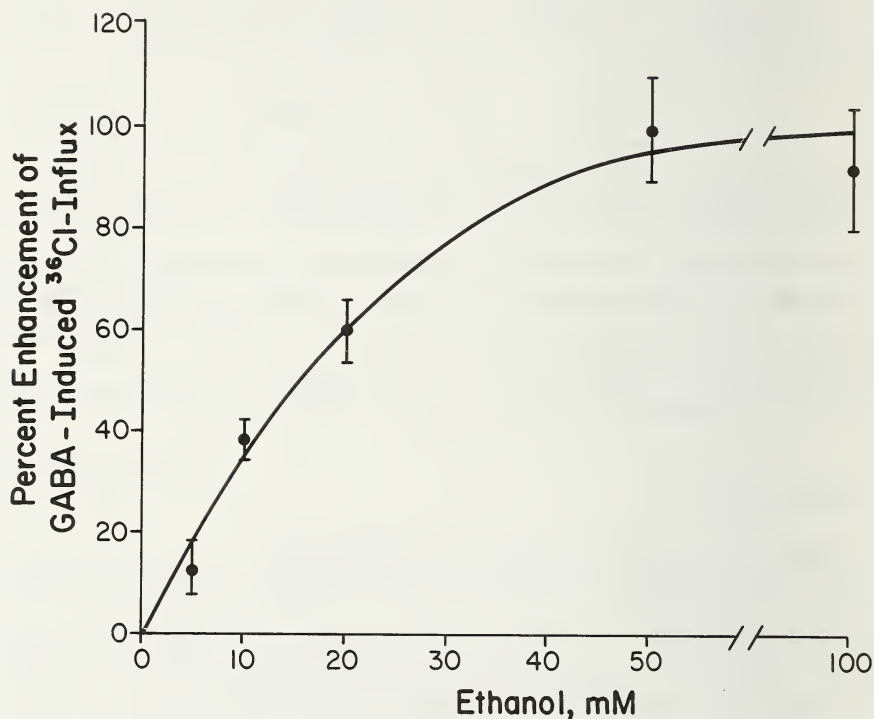


Figure 3.—Concentration-dependent effect of ethanol on GABA ($10\ \mu\text{M}$)-induced ^{36}Cl -influx. The effect of ethanol (5-100 mM) was measured in the absence and presence of GABA ($10\ \mu\text{M}$). The values represent the percentage of increase over the effect of GABA (38.5 ± 3.1 , mean \pm S.E., $n=3$), which was normalized to 100%. The values represent the mean \pm S.E. of three experiments done in triplicate (from Mehta and Ticku 1988).

Both the potentiating and direct effects were specific for GABA_A-gated Cl⁻ channels but not for glycine and were blocked by bicuculline and picrotoxin (Mehta and Ticku 1988). The inverse agonists Ro 15-4513, FG 7142, and methyl 6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM) also blocked this effect; the fact that this effect was reversed by Ro 15-1788 suggests the involvement of the same GABA-gated Cl⁻ channels in the action of ethanol. Ethanol shifted the GABA dose-response curve to the left by enhancing the affinity of GABA by twofold (Mehta and Ticku 1988). Thus, a GABA-benzodiazepine receptor-mediated Cl⁻ transport mechanism, possibly a chloride channel modulatory function, would explain some of the pharmacologic actions of ethanol.

Ro 15-4513, an azido analog of the classical benzodiazepine receptor antagonist Ro 15-1788 with an ability to bind to benzodiazepine receptors, is reported to reverse several of the acute behavioral and toxic effects of ethanol in mice and rats (Bonetti et al. 1985; Suzdak et al. 1986a; Fadd et al. 1987; Ticku and Kulkarni 1988). It also antagonized ethanol-stimulated chloride uptake in isolated brain vesicles in a benzodiazepine receptor antagonist-sensitive manner, an action not shared by other inverse agonists (Suzdak et al. 1986a). However, in C57BL/6J mouse spinal cord cultured neurons, Ro 15-4513 and FG 7142 blocked the GABA-enhancing and direct effects of ethanol on ³⁶Cl⁻ influx. Furthermore, these partial inverse agonists exhibited inverse agonistic activity in the ³⁶Cl⁻ influx assay in cultured neurons (Mehta and Ticku 1988). These observations, although supporting the view of GABA_A receptor-mediated action of ethanol and its reversal by Ro 15-4513, question the selectivity of Ro 15-4513 as a specific antagonist of ethanol action. Several recent behavioral observations support such a view (Lister 1987; Misslin et al. 1988; Britton et al. 1988).

Though devoid of any action per se, Ro 15-4513 significantly reversed the protective effect of ethanol against the tonic extensor phase of bicuculline-induced convulsions. The protective effect against mortality was also significantly reversed. Unlike the case with bicuculline convulsions, Ro 15-4513 reversed the protective effect of ethanol against picrotoxin-induced mortality up to 50 percent only (Kulkarni and Ticku in press). The facts that Ro 15-4513 produced a preferential reversal of ethanol action for bicuculline as compared with picrotoxin and that this effect was sensitive to blockade by

Ro 15-1788, a benzodiazepine receptor antagonist, suggested that Ro 15-4513 acted at the benzodiazepine receptor site. Moreover, both Ro 15-4513 and FG 7142 elicited proconvulsant effect against bicuculline but not against picrotoxin, which may undermine its potential clinical application (Lister and Nutt 1987; Ticku and Kulkarni 1988).

In a chronic ethanol treatment and ethanol withdrawal study, the inverse agonist Ro 15-4513 showed different profile of action. It produced proconvulsant action against PTZ in the withdrawal groups; interestingly, a higher dose could induce clonic-tonic seizures per se in these animals. This response was blocked by both diazepam and Ro 15-1788 (Mehta and Ticku 1989). This observation strengthens the notions not only that chronic ethanol down regulates the GABA_Aergic system during withdrawal but also that the sensitivity of the receptors, particularly the inverse agonist binding sites, may also be altered. The binding studies indicated that chronic ethanol increases selectively the number of receptor sites for [³H]Ro 15-4513 in rat cerebral cortex and cerebellum (Mhatre et al. 1988). The specific binding of benzodiazepine agonists and antagonists was not altered under identical assay conditions (table 4). We have noted similar observations in cultured spinal cord neurons (unpublished observation). Thus, in behavioral, pharmacologic, and biochemical studies, we observe that Ro 15-4513 preferentially reverses the effects of ethanol that are mediated via the GABA_Aergic system. These results suggest a unique interaction of ethanol with Ro 15-4513 binding sites in the central nervous system. Our studies draw indirect support from the reports of Little et al. (1988), who have recently demonstrated that chronic benzodiazepine treatment increases the effects of FG 7142 and produces full convulsions 24 hr after withdrawal from chronic flurazepam treatment.

In summary, on the basis of these behavioral, biochemical, and functional studies, it is concluded that ethanol potentiates GABA_Aergic transmission in cultured spinal cord neurons through GABA_A-gated Cl⁻ channels. The behavioral and biochemical effects are attributed to the GABA_Aergic action of ethanol, and the partial inverse agonist Ro 15-4513 preferentially reverses some of the effects, including GABA-gated Cl⁻ influx caused by ethanol. Chronic treatment with ethanol seems to alter selectively the binding sites for Ro 15-4513 on the GABA_A-benzodiazepine ionophore-receptor complex, and

these pharmacodynamic changes in the supramolecular complex may have far-reaching consequences in explaining the molecular mechanism of the action of ethanol.

Summary

The interaction of ethanol with the γ -aminobutyric acid (GABA) system was investigated by using behavioral, biochemical, and functional assay approaches. The potential of the imidazobenzodiazepine Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-OxO-4H-imidazo [1,5 α][1,4]-benzodiazepine-3-carboxylate) as an ethanol antagonist in these studies was also studied. Ethanol (5-100 mM) potentiated the effect of GABA on $^{36}\text{Cl}^-$ influx, and at concentrations of ≥ 50 mM it directly activated Cl^- channels in cultured spinal cord neurons. Both the enhancing and direct effects of ethanol on $^{36}\text{Cl}^-$ influx were blocked by GABA antagonists such as bicuculline and picrotoxin and also by the inverse agonists Ro 15-4513 and FG 7142. The ethanol-induced anticonvulsant effect was preferentially reversed by Ro 15-4513 in rats in a Ro 15-1788-sensitive manner. In the alcohol withdrawal group, Ro 15-4513 produced convulsions per se and potentiated the effects of pentylenetetrazol, an action again blocked by diazepam and Ro 15-1788. Chronic ethanol treatment also increased selectively the B_{max} for [^3H]Ro 15-4513 sites in cortex and cerebellum and in cultured spinal cord neurons. Taken together, these behavioral, binding, and functional studies demonstrate that ethanol facilitates GABA_Aergic transmission through GABA-gated Cl^- channels and that Ro 15-4513, through a specific and unique receptor mechanism, reverses some of the behavioral and biochemical effects of ethanol associated with the GABA_A receptor system.

References

- Allan, A.M., and Harris, A.R. Gamma-aminobutyric acid and alcohol actions: Neurochemical studies of long sleep and short sleep mice. *Life Sci* 39:2005-2015, 1986.

- Bonetti, E.P.; Burkward, W.P.; Gabl, M.; and Mohler, H. The partial inverse benzodiazepine antagonist Ro 15-4513 antagonizes acute ethanol effects in mice and rats. *Br J Pharmacol* 86:463, 1985.
- Britton, K.T.; Ehlers, C.L.; and Koob, G.F. Is ethanol antagonist Ro 15-4513 selective for ethanol? *Science* 239:648-649, 1988.
- Cott, J.; Carlson, A.; Engel, J.; and Lindquist, M. Suppression of ethanol-induced locomotor stimulation by GABA-like drugs. *Naunyn-Schmiedeberg's Arch Pharmacol* 295:203-209, 1976.
- Fadd, F.; Mosca, E.; Colombo, G.; and Gessa, G.L. Protection against ethanol mortality in rats by imidazobenzodiazepine Ro 15-4513. *Eur J Pharmacol* 136:265-266, 1987.
- Harris, R.A., and Allan, A.M. Functional coupling of GABA receptors to chloride channels in brain membranes. *Science* 228:1108-1110, 1985.
- Hunt, W.A. The effect of ethanol on GABAergic transmission. *Neurosci Biobehav Rev* 7:87-95, 1983.
- Kulkarni, S.K., and Ticku, M.K. Ro 15-4513 but not FG 7142 reverses anticonvulsant effects of ethanol against bicuculline- and picrotoxin-induced convulsions in rats. *Pharmacol Biochem Behav* 32:233-240, 1989.
- Liljequist, S.; Culp, S.; and Tabakoff, B. Effects of ethanol on the binding of [³⁵S]t-butylbicyclophosphorothionate to mouse brain membranes. *Life Sci* 38:1931-1939, 1986.
- Liljequist, S., and Engel, J. Effects of GABAergic agonists and antagonists on various ethanol-induced behavioral changes. *Psychopharmacology* 78:71-75, 1982.
- Lister, R.G. The benzodiazepine receptor inverse agonists FG 7142 and Ro 15-4513 both reverse some of the behavioral effects of ethanol in a holeboard test. *Life Sci* 41:1481-1489, 1987.
- Lister, R.G., and Nutt, D.J. Is Ro 15-4513 a specific alcohol antagonist? *Trends Neurosci* 10:223-225, 1987.

- Little, H.J.; Gale, R.; Sellars, N.; Nutt, D.J.; and Taylor, S.C. Chronic benzodiazepine treatment increases the effects of the inverse agonist FG 7142. *Neuropharmacology* 27:383-389, 1988.
- Mehta, A.K., and Ticku, M.K. Characteristics of flunitrazepam binding in intact cultured spinal cord neurons and its modulation by GABAergic drugs. *J Neurochem* 49:1491-1497, 1987.
- Mehta, A.K., and Ticku, M.K. Ethanol potentiation of GABAergic transmission in cultured spinal cord neurons involves α -aminobutyric acid_A-gated chloride channels. *J Pharmacol Exp Ther* 246:558-564, 1988.
- Mehta, A.K., and Ticku, M.K. Chronic ethanol administration alters the behavioral effects of Ro 15-4513, a partially negative ligand for benzodiazepine binding sites. *Brain Res* 489:93-100, 1989.
- Mendelson, W.B.; Martin, J.V.; Wagner, R.; Roseberry, C.; Skolnick, P.; Weissman, B.A.; and Squires, R. Are the toxicities of pentobarbital and ethanol mediated by the GABA-benzodiazepine receptor-chloride ionophore complex? *Eur J Pharmacol* 108:63-70, 1985.
- Mhatre, M.; Mehta, A.K.; and Ticku, M.K. Chronic ethanol administration increases the binding of the benzodiazepine inverse agonist and alcohol antagonist [³H]Ro 15-4513 in rat brain. *Eur J Pharmacol* 153:141-145, 1988.
- Misslin, R.; Belzung, C.; and Vogel, E. Interaction of Ro 15-4513 and ethanol on the behavior in mice: Antagonistic or additive effects. *Psychopharmacology* 94:392-396, 1988.
- Nestoros, J.N. Ethanol specifically potentiates GABA-mediated neurotransmission in feline cerebral cortex. *Science* 209:708-710, 1980.
- Olsen, R.W. Drug interactions at the GABA receptor-ionophore complex. *Annu Rev Pharmacol* 22:245-277, 1982.
- Rastogi, S.K.; Thyagarajan, R.; Clothier, J.; and Ticku, M.K. Effects of chronic treatment of ethanol on benzodiazepine and picrotoxin sites on the

- GABA receptor complex in regions of the brain of the rat. *Neuropharmacology* 25:1179-1184, 1986.
- Rastogi, S.K., and Ticku, M.K. Involvement of a GABAergic mechanism in the anticonvulsant effect of pentobarbital against maximal electroshock-induced seizures in rats. *Pharmacol Biochem Behav* 22:141-146, 1985.
- Rastogi, S.K., and Ticku, M.K. Anticonvulsant profile of drugs which facilitate GABAergic transmission on convulsions mediated by a GABAergic mechanism. *Neuropharmacology* 25:175-185, 1986.
- Suzdak, P.; Glowa, J.R.; Crawley, J.N.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. A selective imidazodiazepine antagonist of ethanol in the rat. *Science* 234:1243-1247, 1986a.
- Suzdak, P.D.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. Ethanol stimulates γ -aminobutyric acid receptor-mediated chloride transport in rat brain synaptoneurosome. *Proc Natl Acad Sci USA* 83:4071-4075, 1986b.
- Thampy, K.G., and Barnes, E.M., Jr. γ -Aminobutyric acid-gated chloride channels in cultured cerebral neurons. *J Biol Chem* 259:1753-1757, 1984.
- Thyagarajan, R., and Ticku, M.K. The effects of in vitro and in vivo ethanol administration of [35 S]t-butylbicyclophosphorothionate binding in C57 mice. *Brain Res Bull* 15:343-345, 1985.
- Ticku, M.K. Benzodiazepine-GABA receptor ionophore complex: Current concepts. *Neuropharmacology* 22:1459-1470, 1983.
- Ticku, M.K., and Kulkarni, S.K. Molecular interactions of ethanol with GABAergic system and potential of Ro 15-4513 as an ethanol antagonist. *Pharmacol Biochem Behav* 30:501-510, 1988.
- Ticku, M.K.; Lowrimore, P.; and Lehoullier, P. Ethanol enhances GABA-induced $^{36}\text{Cl}^-$ influx in primary spinal cord cultured neurons. *Brain Res Bull* 17:123-126, 1986.

Electrophysiologic Interactions of Ethanol With Ro 15-4513 and γ -Aminobutyric Acid Mechanisms on Rat and Human Neurons¹

*Michael R. Palmer*²

Introduction

Although the central nervous system mechanisms of ethanol-induced changes in behavior remain largely unknown, ethanol has been reported to potentiate some of the behavioral effects thought to be mediated by γ -aminobutyric acid

¹The following people contributed technically and/or scientifically to the data presented here: A.-C. Granholm, Department of Cell Biology, University of Linköping Health Science Center, Linköping, Sweden; Å. Seiger, Department of Geriatrics, Huddinge Hospital, Stockholm, Sweden; M. Bygdeman, M. Eriksson-Nilsson, L. Olson, P. Stieg, and I. Strömberg, Departments of Histology and Neurobiology and of Obstetrics and Gynecology, Karolinska Institute and Karolinska Hospital, Stockholm, Sweden; and J. T. Harlan, Craig G. van Horne, R. A. Harris, B. J. Hoffer, and Elizabeth A. Moore, Department of Pharmacology, University of Colorado Health Science Center, Denver, CO. The work summarized in this presentation was supported by Public Health Service grants AA05915, AA03527, and AA00102. Dr. Palmer is supported by an ADAMHA research scientist development award.

²Department of Pharmacology, University of Colorado Health Sciences Center, Denver, CO 80262.

(GABA) synapses in the brain (Cott et al. 1974; Liljequist and Engel 1982; Martz et al. 1983). Furthermore, ethanol shares muscle relaxant, antianxiety, and sedative-hypnotic properties with agents that potentiate GABA_A receptor function, such as barbiturates and benzodiazepines (Burch and Ticku 1980; Skolnick and Paul 1981). More recent evidence suggests that ethanol can augment the opening of Cl⁻ channels by GABA agonists (Allan and Harris 1985, 1986; Suzdak et al. 1986a,b; Ticku et al. 1986), and a recent review of literature concludes that at least some ethanol actions may be mediated through an enhancement of the function of the benzodiazepine-sensitive GABA_A-Cl⁻ channel complex (Allan and Harris 1987). Consistent with the hypothesis that some ethanol effects on brain tissue might be mediated through a GABA mechanism of action, ethanol has been reported to potentiate GABA effects on neurons in chick spinal cord and cat neocortex (Celentano et al. 1988; Nestoros 1980). Furthermore, GABA_A antagonists have been reported to block depressions of neuronal activity in rat substantia nigra by systemic ethanol (Mereu and Gessa 1985). In contrast, however, the electrophysiologic effects of GABA in hippocampus and in cerebellum have been reported to be either unaffected or antagonized by ethanol (Bloom and Siggins 1987; Carlen et al. 1982; Harris and Sinclair 1984; Siggins et al. 1987). The imidazobenzodiazepine Ro 15-4513, a partial inverse agonist at the benzodiazepine receptor on the GABA_A-Cl⁻ complex (Bonetti et al. 1984; Nutt and Lister 1987; Polc et al. 1985), has been reported to antagonize some of the behavioral, biochemical, and physiologic effects of ethanol (Bonetti et al. 1985; Hoffman et al. 1987; Nutt and Lister 1987; Polc 1985; Suzdak et al. 1986a). However, there is some controversy as to whether the Ro 15-4513 antagonism of ethanol is mediated through a benzodiazepine mechanism, and it is not known whether Ro 15-4513 directly antagonizes ethanol or, alternately, whether this inverse benzodiazepine agonist has more complex interactions with ethanol through separate but interactive physiologic mechanisms. Finally, to date this putative ethanol antagonist has not been tested in human tissue because of potential toxic side effects that have not yet been completely characterized.

This presentation reviews our recent *in vivo* electrophysiologic experiments, which explore the involvement of GABA mechanisms in ethanol-induced alterations of the electrophysiologic activity of single cerebellar Purkinje neurons *in situ* and study the ability of two inverse agonists, Ro 15-4513 and

FG 7142, to alter the dose-response relationship of Purkinje neuron responses to locally applied ethanol in both rat and human neurons. The human experiments were carried out by using a unique preparation that allows invasive electrophysiologic studies to be performed at the cellular level on human central neurons *in vivo*: xenografts of cerebral and cerebellar human cortices grafted to the anterior eye chamber of athymic nude rats.

Methods

Xenograft Methods

To generate the xenografts, cerebral and cerebellar cortical fragments were dissected from aborted human fetal fragments and subsequently inserted into the anterior eye chamber of adult athymic rats as previously described (Olson et al. 1983, 1987; Bickford-Wimer et al. 1987; Granholm et al. 1989). Fetal brain tissue was obtained in Sweden after termination of first-trimester pregnancies in healthy women admitted to the hospital for elective abortions. Nude athymic rats were used because they lack the ability to produce an immune response (Festing et al. 1978; Vos et al. 1980), and therefore xenografts of human brain tissue to these animals are not rejected (Hall et al. 1987). The grafted tissue was allowed to mature for 3-11 months before electrophysiologic studies. The study was approved by the Regional Ethical Committee of the Karolinska Hospital, and all experiments on this tissue conformed to guidelines of the Swedish Medical Council and the U.S. Public Health Service.

Electrophysiology Methods

The experimental animals for the *in situ* experiments and the host recipient of the xenografts were male Sprague-Dawley rats weighing 200-450 g. For physiologic experiments, these animals were anesthetized with 1.25 g/kg of urethane, intubated, and placed in a stereotaxic frame. Body temperature was monitored by a rectal thermistor probe and maintained at 37 °C by a heating pad. We have previously published detailed electrophysiologic methods for recording single-action potentials with single and multibarrel glass micropipettes from *in situ* cerebellar Purkinje as well as from brain grafts *in oculo* (Palmer et al. 1986, 1988; Granholm and Palmer 1988). Fifty

percent depression of the spontaneous firing rate was defined as the psi-sec (pounds per square inch x second) dose required to elicit a 30-70 percent response. This response window was used to avoid ceiling and threshold effects (Sorensen et al. 1980). For dose-response curves, the actual depressions observed were used. The actions of the locally applied inverse benzodiazepine agonists far outlasted the duration of their administration in preliminary experiments; therefore, ethanol and GABA effects could be tested long after termination of the antagonist application. Each neuron was required to exhibit a stable firing rate during pre- and post-drug periods, and drug agonist responses were acceptable only if they were repeatable and reversible. Changes in ethanol or GABA effects by antagonists were investigated only after each neuron had fully recovered from any direct solvent effects associated with local antagonist applications.

Using methods that we have previously described in detail, GABA was applied by microiontophoresis (Palmer and Hoffer 1980), while other drugs were locally applied from micropipettes in situ by pressure ejection (Palmer 1982). Drugs were superfused over the in oculo brain grafts as previously described (Granhölm and Palmer 1988).

Results and Discussion

Antagonism of Ethanol by Ro 15-4513 in Rat Brain

In a recent paper (Palmer et al. 1988) we reported finding that the benzodiazepine derivative Ro 15-4513, when applied at maximally effective doses from micropipettes by pressure ejection, antagonized the ethanol-induced depressions in 47 of the 59 neurons studied (figure 1). The average 50 percent ethanol-induced inhibition for these neurons, which was a 47.2 ± 1.3 percent depression in the control epoch, was significantly reduced ($P < 0.001$, paired t test) to an average of 22.7 ± 2.2 percent depression after local Ro 15-4513 applications. The Ro 15-4513-induced antagonism of ethanol-induced depressions typically lasted more than 1 hr; 30 of the 47 neurons sensitive to Ro 15-4513 partially or fully recovered to previous ethanol sensitivities within 2 hr after termination of the Ro 15-4513 application. Similarly, FG 7142, a beta-carboline inverse agonist (Braestrup

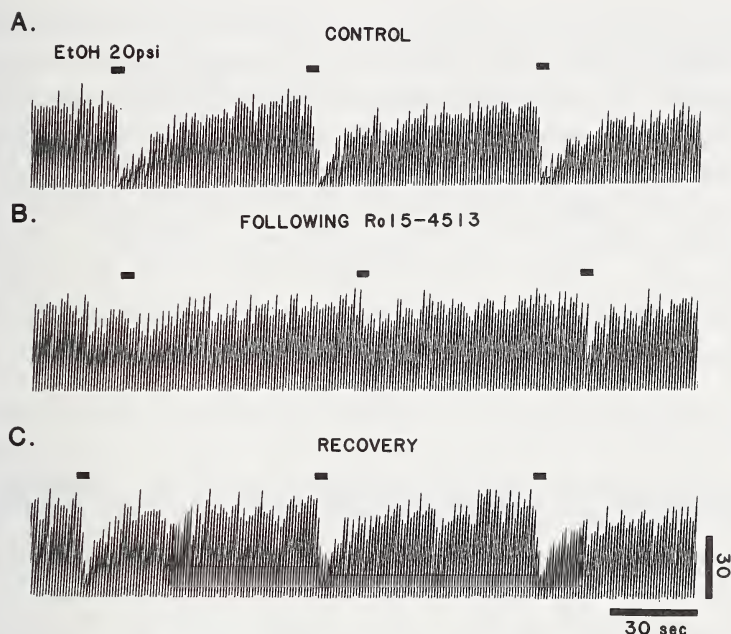


Figure 1.—Antagonism of ethanol-induced depressions by Ro 15-4513: ratemeter records demonstrating the Ro 15-4513 sensitivity of ethanol-induced depressions in spontaneous firing rate of cerebellar Purkinje neurons caused by local pressure ejection applications of ethanol (EtOH) from micropipettes. Local pressure applications of ethanol at 20 psi for 4 s caused reproducible depressions of neuronal firing rate (A). These responses were reduced but not eliminated during and after the local application of Ro 15-4513 (B) but recovered approximately 1.5 hr later (C). In this figure and in figure 2, the horizontal axis represents time, and the vertical axis represents action potentials per second; calibrations are indicated in the lower righthand corner. The length of each bar over the recordings indicates the duration of ethanol ejection; ejection pressure is indicated over the first bar of the figure. (From Palmer, M.R.; van Horne, C.G.; Harlan, T.J.; and Moore, E.A. Antagonism of ethanol effects on cerebellar Purkinje neurons by the benzodiazepine inverse agonists Ro 15-4513 and FG 7142: Electrophysiological studies. *J Pharmacol Exp Ther* 247:(3)1018-1024, 1988. Copyright by the *Journal of Pharmacology and Experimental Therapeutics*.)

et al. 1984), significantly antagonized ($P < 0.001$, paired t test) the depressant effects of ethanol locally applied, using the same paradigm on 15 of the 17 neurons studied. These data suggest that this ethanol interaction might be a general property of benzodiazepine inverse agonists rather than a unique property of Ro 15-4513. In contrast to Ro 15-4513, the maximal effect of FG 7142 was a 90-100 percent antagonism of the 50 percent depressions of neuronal activity induced by local ethanol applications (figure 2). These observations agree with the $\text{GABA}_A\text{-Cl}^-$ channel studies which also suggest that FG 7142 might be more efficacious but less potent than Ro 15-4513 for antagonizing ethanol effects (Harris et al. 1988; Mehta and Ticku 1988).

We observed that the duration of the ethanol antagonism generated by both Ro 15-4513 and FG 7142 was extraordinarily long in comparison with actions of other drugs applied locally to neurons in vivo from micropipettes (Gerhardt and Palmer 1987; Stone 1985). This phenomenon might be related to the high lipid-water partition coefficient of Ro 15-4513 and FG 7142, since other lipid-soluble drugs, such as propranolol and L-phenylisopropyl adenosine (L-PIA), also accumulate in the lipid membrane environment of cells. Since these drugs become highly concentrated (up to 40-fold) in brain tissue (Dunwiddie and Fredholm 1985; Pruett et al. 1980), brief local applications can cause effects that last for more than 1 hr (Brodie et al. 1987; Dunwiddie et al. 1984). Local applications of Ro 15-4513, while causing only small, statistically insignificant increases in the 50 percent effective concentration (EC_{50}) of ethanol dose-response curves, did significantly reduce the maximal response to pressure-ejected ethanol (figure 34). A mean maximal response before antagonist application of 82 ± 5 percent depression was significantly reduced to 25 ± 7 percent depression after Ro 15-4513 application to these neurons ($P < 0.001$, paired t test). Furthermore, when minimally effective low doses of Ro 15-4513 were followed by maximally effective doses, it was consistently observed that the primary effect of the higher Ro 15-4513 dose was a decrease in the maximal response of the ethanol dose-response curve from that recorded after the first Ro 15-4513 application on those neurons (figure 34). This observation may explain our finding that Ro 15-4513 could not completely antagonize the 50 percent ethanol depressions described above. The observed 80 percent decrease in the maximal response to ethanol would result in a concomitant 80 percent reduction of the response to a dose causing 50 percent depressions

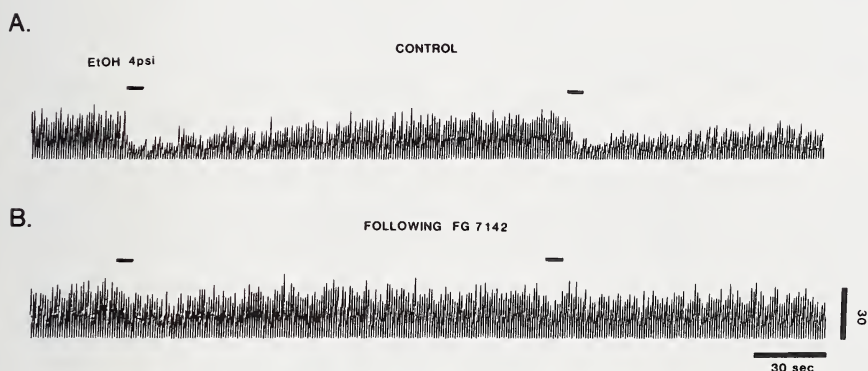


Figure 2.—Antagonism of ethanol (EtOH)-induced depressions by FG 7142: ratemeter record demonstrating the FG 7142 sensitivity of ethanol-induced depressions in spontaneous firing rate of a cerebellar Purkinje neuron caused by local pressure ejection applications of ethanol from micropipettes. Local pressure ejection applications of ethanol at 4 psi for 8 s caused reproducible depressions of neuronal firing rate (*A*). These ethanol-induced depressions were blocked by the local application of FG 7142 (*B*).

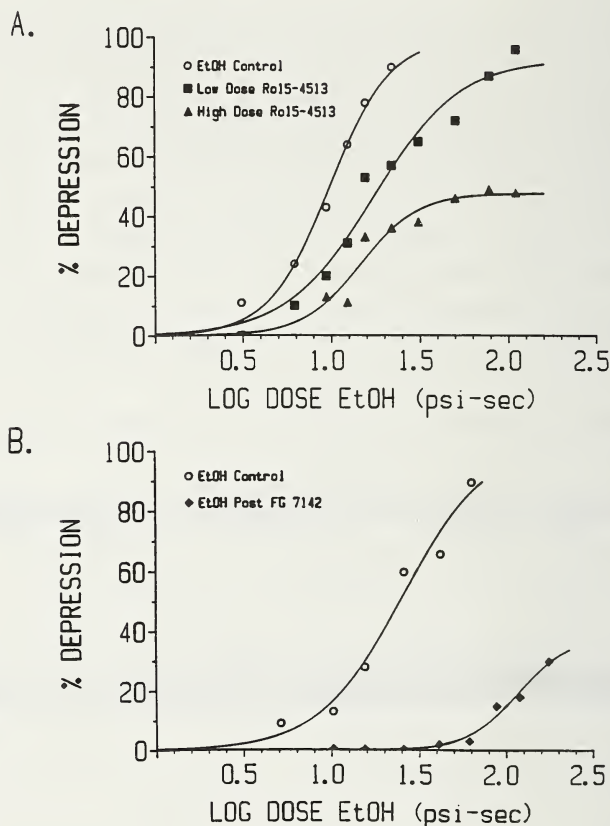


Figure 3.—Sensitivity of ethanol (EtOH)-induced depressions to Ro 15-4513 and FG 7142 in rat brain in vivo: log dose-response curves illustrating the percent depression in spontaneous firing rate due to a range of pressure doses of ethanol before and after the local application of Ro 15-4513 (A) and FG 7142 (B). A low dose of Ro 15-4513 caused a small shift of the ethanol EC_{50} to the right, while a subsequent higher dose of Ro 15-4513 caused the typical large decrease in the maximal response to ethanol. (From Palmer, M.R.; van Horne, C.G.; Harlan, T.J.; and Moore, E.A. Antagonism of ethanol effects on cerebellar Purkinje neurons by the benzodiazepine inverse agonists Ro 15-4513 and FG 7142: Electrophysiological studies. *J Pharmacol Exp Ther* 247:(3)1018-1024, 1988. Copyright by the *Journal of Pharmacology and Experimental Therapeutics*.)

before the antagonist application. Although FG 7142 was less potent than Ro 15-4513 in our *in vivo* system, the FG compound caused a large shift of the ethanol dose-response curve to the right (figure 3B) that was not observed with Ro 15-4513. This shift resulted in a large change in the ethanol EC_{50} on all cells studied ($P < 0.001$) and explains the more efficacious antagonism of ethanol 50 percent responses by FG 7142. The prominent decrease in the maximal response of ethanol dose-response curves caused by the inverse benzodiazepine agonists suggests that this interaction is clearly not mediated by a competitive mechanism. An uncompetitive interaction would be consistent with the hypothesis that Ro 15-4513 and FG 7142 only indirectly interact with ethanol, perhaps through an allosteric mechanism. Indeed, ethanol and inverse benzodiazepine agonists have been proposed to interact with different components of the Cl^- channel macromolecular complex (Lister and Nutt 1987; Nutt and Lister 1987).

In addition to the observed antagonism of ethanol-induced depressions, the inhibitory response to ethanol became an excitatory response during or after Ro 15-4513 applications on 10 of 59 neurons. Similar effects were observed after FG 7142 applications on 5 of 17 cells studied. In these experiments, a given ethanol dose for producing pure inhibitions before application of Ro 15-4513 or FG 7142 produced pure excitations after application of these partial inverse benzodiazepine agonists. The ethanol-induced excitations gradually subsided, and the ethanol-induced inhibition of neuronal firing rates returned as these cells appeared to recover from the effects of antagonist application. Previous reports demonstrate that ethanol can cause excitations of the fire rates of cerebellar Purkinje neurons when administered systemically (Rogers et al. 1980; Sorenson et al. 1981) or when perfused over cerebellar tissue *in vitro* (Basile et al. 1983; Davidoff 1973; George and Chu 1984; Seil et al. 1977; Sinclair and Lo 1981). We have previously found that the more slowly developing depressant response to ethanol can surmount an initial excitation to lower doses of ethanol applied both by superfusion in the *in oculo* cerebellar graft (Palmer et al. 1982) and by local pressure application from micropipettes to Purkinje neurons *in situ*. Perhaps the excitations observed in the present study represent Ro 15-4513-insensitive ethanol responses that are usually masked by the more dominant depressant actions of ethanol in the cerebellum. Alternately, Harris and collaborators (1988) have found that ethanol potentiates the inverse agonist actions of Ro

15-4513 and FG 7142. In our system, such an effect could result in an inverse agonist-induced reduction of tonic GABAergic input to Purkinje neurons. The resulting disinhibition would likely be manifested as an apparent excitation of neuronal activity.

It is interesting that ethanol-induced depressions were more completely antagonized ($P < 0.01$) by Ro 15-4513 in those studies in which GABA was alternately applied with ethanol than in those in which ethanol was applied alone. It is possible that the ethanol effects studied here are the result of an allosteric potentiation of the GABA response mechanism. Thus, the expression of the ethanol effect in vivo might be dependent on an intact GABA response without necessitating a direct interaction of ethanol with the GABA mechanism of action. If so, then allosteric interactions between the Ro 15-4513 and ethanol mechanisms of action might result in a decreased influence of ethanol on the cellular mechanism activated by GABA.

Antagonism of Ethanol by Ro 15-4513 in Human Brain Xenografts

Preliminary studies to be published elsewhere indicate that Ro 15-4513 effectively antagonizes ethanol effects in human tissue as well. After 3-11 months of maturation in oculo, recordings were made from single neurons in human xenografts that were either derived from the cerebellar anlage or dissected from neocortical tissue. Superfusion of ethanol predominantly elicited inhibitions of neuronal firing, although excitations were occasionally observed at doses of 1-10 mM. The neocortical transplants could be divided into two groups with respect to neuronal ethanol sensitivity (figure 4). The EC_{50} s for ethanol-induced slowing of discharge in preliminary studies on human cerebellar grafts suggested two distinct populations as well. However, the ethanol sensitivities of different neurons in any one graft and of all neurons derived from a given fetus were consistently within the same sensitivity range. The 30 mM sensitivity of the larger group, which translates into 138 mg percent, falls into the dose range in humans who are displaying ethanol-induced ataxia. The fact that this reflects a much higher ethanol sensitivity than is found in most strains of rodents emphasizes the need to establish "physiologically relevant ethanol levels" separately for each animal model rather than assuming ethanol levels that are relevant for human behavior

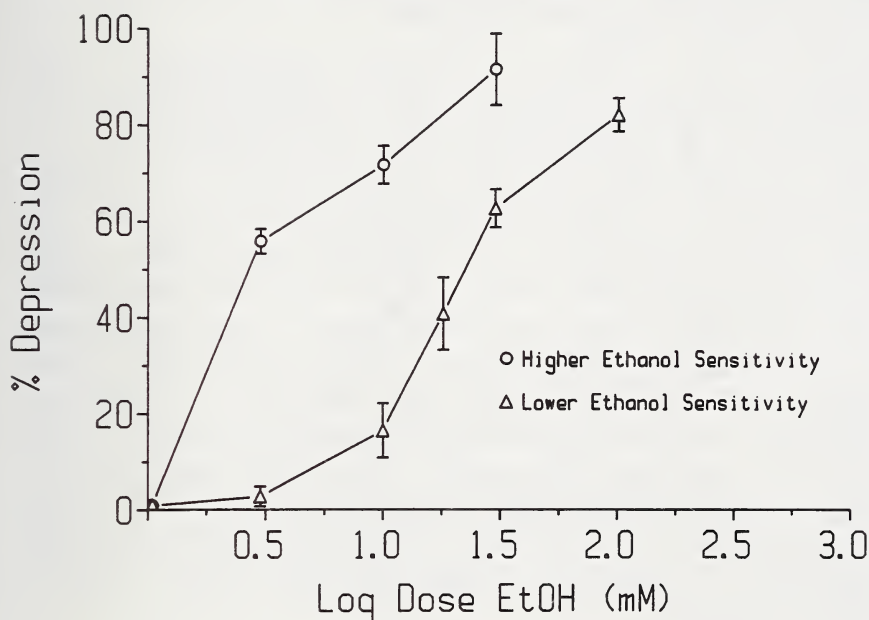


Figure 4.—Preliminary data illustrating the depressant effect of superfused ethanol (EtOH) on the firing rate of single neurons in human xenografts in oculo. The data are represented as log dose-response curves of the depressant effect of superfused ethanol in all the xenografts studied to date. These data indicate two dissimilar ethanol sensitivities found among the human neocortical xenografts. The ordinate represents the percent depression of firing rate \pm standard error of the mean caused by a given dose of superfused ethanol, and the superfused ethanol dose is indicated on the abscissa. Neurons in the high- and low-sensitivity groups were averaged separately.

are generalized to various animals as well. Our observation that the grafts fall into two distinct categories in terms of ethanol sensitivity may reflect genetic differences between the acute ethanol sensitivity of the donor tissue, or it may reflect the influences of early prenatal exposure to ethanol or other drugs. Unfortunately, we do not have access to enough donor background history to approach these questions at present.

Similar to findings in animals, the depressant effects of ethanol in this xenograft model could be antagonized by the benzodiazepine inverse agonist Ro 15-4513 in all neocortical or cerebellar transplants tested (figure 5). Unlike the ethanol antagonism by Ro 15-4513 described above in rat brain, the application of Ro 15-4513 (1 μ M) shifted the ethanol dose-response curve to the right by more than one order of magnitude. This increased ethanol EC₅₀ lasted for 1.5-2 hr after cessation of the Ro 15-4513 application in these grafts, after which the response to ethanol returned to control levels (figure 5). This finding might suggest that Ro 15-4513 is a more potent ethanol antagonist in human central nervous system tissue than it is in the rat. Furthermore, we saw no evidence of seizures induced by 1 μ M Ro 15-4513 in any of the neocortical grafts studied, which might suggest that the epileptogenic potency of this agent is well below its potency as an ethanol antagonist. However, this latter observation may well depend on the seizure susceptibility of transplants derived from a given brain area and will require further investigation.

Interaction of Ethanol with GABA_A Mechanisms in Cerebellum

In an effort to determine the role of GABA synapses in the observed ethanol effects, we studied interactions between the effects of local pressure ejections of ethanol and microiontophoretic applications of GABA onto rat cerebellar Purkinje neurons in situ. The inhibitions of cerebellar Purkinje neuron firing rate caused by local applications of GABA can be blocked by the GABA_A antagonists bicuculline (Curtis and Felix 1971; Woodward et al. 1971) and picrotoxin (Geller et al. 1978); as described below, we also find that bicuculline completely antagonizes the depressant effects of locally applied GABA. These data suggest that the depressant GABA effects on these

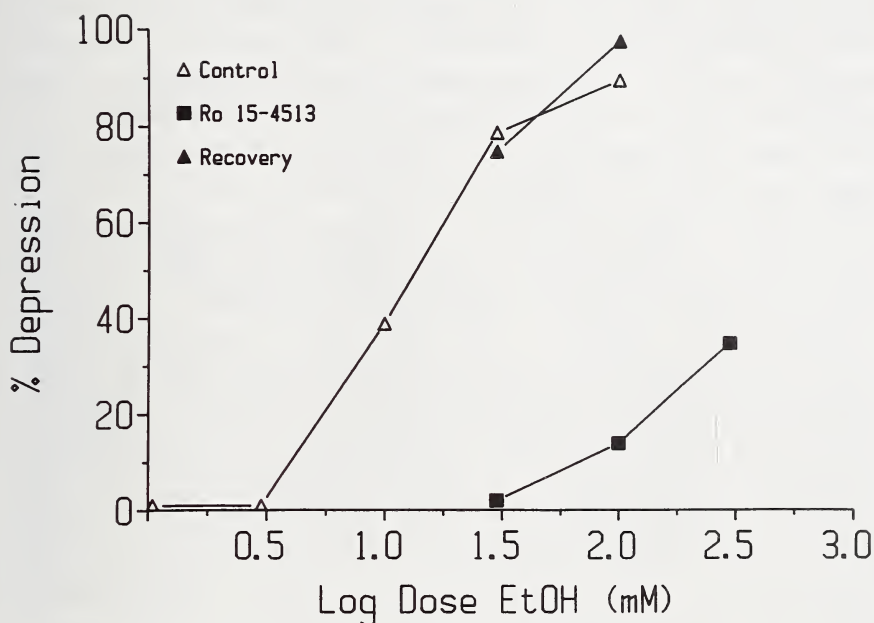


Figure 5.—Preliminary data illustrating the antagonism of the depressant electrophysiologic effects of ethanol (EtOH) by Ro 15-4513 in a human neocortical xenograft. This figure represents ethanol log dose-response curves from a single neocortical neuron in a human xenograft and illustrates a representative neuronal sensitivity to superfused ethanol before (▲) and immediately after (■) the superfusion of 1 M Ro 15-4513. The antagonist caused a large shift of the ethanol dose-response curve to the right. The two points represented by the closed triangles represent recovery of ethanol sensitivity 2 hr after cessation of the Ro 15-4513 application. The ordinate represents the percent depression of firing rate caused by a given dose by superfused ethanol, and the superfused ethanol dose is indicated on the abscissa.

neurons are mediated by a GABA_A mechanism. In our experiments, local applications of both ethanol and GABA caused reversible, dose-dependent depressions of spontaneous firing rates of all neurons studied. Local pressure applications of ethanol, which were just threshold for slowing neuronal activity and which were administered constantly during several GABA applications, did not consistently potentiate GABA effects but did reduce the size of the GABA effects on about half of the cells studied. This observation is consistent with data from other laboratories indicating that ethanol does not increase the size of GABA effects in either cerebellum or hippocampus (Bloom and Siggins 1987; Carlen et al. 1982; Harris and Sinclair 1984; Siggins et al. 1987). Similar to Farb and co-workers (Celentano et al. 1988), however, we also find that when applied in brief pulses rather than as a sustained application, ethanol will cause a potentiation of GABA-induced depressions. We found that brief 5- to 15-s local applications of ethanol between sequential GABA responses potentiated the subsequent GABA effects on one-third of the cells studied. Thus, consistent with the earlier report, we concluded that the protocol of ethanol application does appear to be important for the elucidation of this ethanol effect. The ethanol-induced potentiations of the GABA effects observed lasted for 10-15 min but were quantitatively quite modest. Even though the size of the potentiation was small, it should be noted that the reported increase in GABA-stimulated Cl⁻ flux is also relatively small (Allan and Harris 1986; Mehta and Ticku 1988; Suzdak et al. 1986*a,b*; and Ticku et al. 1986). Even so, not all neurons were affected this way, and when observed, the increase in the GABA effect was too small to be responsible for the prominent depression of neuronal activity that is consistently observed on every Purkinje neuron in response to local ethanol applications. Furthermore, neither brief nor continuous GABA applications potentiated ethanol-induced 50 percent depressions of neuronal firing rate. Finally, it is difficult to envision how systemic doses of ethanol could effectively mimic this potentiation, since sustained applications of ethanol were not effective. It is possible that the GABA response is already nearly maximally potentiated by the urethane anesthetic, and this possibility should be investigated. However, the depressant effects that we observe to local ethanol applications are also observed in the same anesthetized animals. Thus, these data suggest that the depressant ethanol effects on single Purkinje neurons in situ are not simply mediated by a GABA mechanism of action.

In apparent contrast to the potentiation data presented above, and consistent with the growing hypothesis that ethanol effects are mediated through a GABA_A mechanism, we found that bicuculline, when pressure ejected from the same micropipette as either GABA or ethanol, completely and reversibly antagonized the depressant effects of both GABA and ethanol on Purkinje neuron firing rate in situ. The bicuculline-induced antagonism of depressant responses to ethanol applications required 15-20 min to subside and far outlasted the antagonisms of GABA effects even on the same cell. These data clearly suggest that a GABA_A mechanism is involved in the expression of that ethanol response. It is possible, however, that bicuculline has effects in addition to a GABA_A antagonism. GABA antagonists have previously been reported to antagonize the effects of both norepinephrine in cat thalamus (Phillis and Tebecis 1967) and serotonin in cat neocortex (Straughan and Watson 1972). Furthermore, the bicuculline reversal of depressant effects could be due simply to an increased level excitability of the neurons. However, similar to a previous study in rat brainstem (Dray 1975), we find that bicuculline does not alter the depressant effects of local applications of norepinephrine at doses that antagonized ethanol effects on the same cells. Furthermore, similar bicuculline applications did not alter the depressant effects of locally applied lidocaine on Purkinje neurons. Thus, the ethanol antagonism caused by the GABA_A antagonist bicuculline appears to be relatively selective.

Our finding that bicuculline blocks the electrophysiologic effects of ethanol, together with previous biochemical reports demonstrating that ethanol has actions at the GABA_A-Cl⁻ channel (Allan and Harris 1986; Mehta and Ticku 1988; Suzdak et al. 1986b; Ticku et al. 1986), suggests the GABA_A mechanism is involved in the ethanol response. However, since we are unable to produce large, routine potentiations of GABA effects by ethanol, we feel that it is more likely that our data indicate that a GABA_A mechanism is playing a permissive role in the ethanol response. That is, a GABA_A mechanism may not mediate the ethanol response, but activation of the GABA_A mechanism may be a prerequisite for expression of the ethanol-induced depressions. The potentiation of synaptic GABA levels, for example, through a GABA reuptake blockade, would fit with this hypothesis. Such a mechanism might also be consistent with our finding that the partial antagonism of ethanol in

the cerebellum by the inverse benzodiazepine agonists Ro 15-4513 and FG 7142 is not competitive. Since the latter drugs act by regulating Cl^- channel function via allosteric interactions with other subunits of the $\text{GABA}_A\text{-Cl}^-$ receptor-channel complex (Allan and Harris 1986; Harris et al. 1988; Mehta and Ticku 1988; Suzdak et al. 1986a), they would not be expected to competitively antagonize the effects of either direct or indirect GABA agonists. An alternate explanation for our data may be that ethanol and GABA do not share a common mechanism of action on Purkinje neurons, but that their mechanisms do have physiologic interactions. Similarly, at least one previous biochemical study suggests that while ethanol alone does not increase Cl^- flux through the GABA_A channel, it does increase the size of the channel response to GABA agonists (Allan and Harris 1986).

Summary

We have found that the partial inverse benzodiazepine agonists Ro 15-4513 and FG 7142 antagonize the depressant, electrophysiologic effects of locally applied ethanol in the cerebellum. FG 7142 was more efficacious but less potent than Ro 15-4513. We also found that the antagonism of ethanol observed in our system was not competitive. Similarly, ethanol-induced depressions of human neurons in cerebellar and cerebral cortex xenografts from first-trimester fetal tissue fragments placed in oculo into adult rat hosts could be antagonized by administration of the benzodiazepine inverse agonist Ro 15-4513. As with the rat studies, the ethanol antagonism by this inverse benzodiazepine agonist was not competitive. Our observation that ethanol and inverse benzodiazepine agonists have interactions that are not competitive might suggest that these two drugs act through separate but interactive mechanisms to produce the observed ethanol antagonism. If such independent interactions were mediated at different sites on a given macromolecular complex such as the $\text{GABA}_A\text{-Cl}^-$ channel, then one might be expected to find allosteric interactions between those interactive sites as well as with the functional response of the complex to GABA activation. Indeed, this hypothesis is consistent with the recent finding of Harris and collaborators (1988) that ethanol potentiates the inverse agonist actions of Ro 15-4513 and FG 7142. Inconsistent with this hypothesis, however, we were unable to find large ethanol-induced potentiations of GABA effects on all neurons that showed depressant responses to ethanol administration in rat cerebellum.

However, we did find that the GABA_A antagonist bicuculline blocks the depressant effects of ethanol on the same neurons. We concluded that ethanol and GABA do not have direct interactions at the GABA_A receptor site but that the GABA_A mechanism does play a permissive role in the ethanol-induced depressions of cerebellar Purkinje neurons. Thus, although a postsynaptic GABA_A mechanism may not be the primary site of action at which ethanol causes depressant electrophysiologic responses of neurons to ethanol application, activation of the GABA_A receptor may be required to make cerebellar Purkinje neurons responsive to the depressant actions of ethanol.

The in oculo brain graft, with which neuronal circuits can be generated in isolation from other brain inputs, may prove to be an effective in vivo model system for studying the involvement of GABA and other putative neurotransmitters in the trans-synaptic effects of superfused ethanol on a given neuronal circuit. Furthermore, using the human in oculo xenograft model, we also found that there were at least two populations of human neurons as judged by acute ethanol sensitivity. The sensitivities of all neurons in a given xenograft corresponded and appeared to be dependent on the fetus of origin. Thus, in future studies, we may be able to relate fetal neuronal ethanol sensitivity to donor genetic factors and/or to prenatal ethanol exposure. Furthermore, the xenografts may be a unique model for fetal alcohol syndrome, since isolated neuronal circuitries can be prenatally and/or postnatally exposed to ethanol under various environmental conditions. Finally, it has been suggested that Ro 15-4513 may cause convulsant side effects that could limit its clinical usefulness. The in oculo brain transplant model may represent a unique experimental preparation in which the potency of alcohol antagonists can be directly compared in vivo with their proconvulsant properties.

References

- Allan, A.M., and Harris, R.A. Ethanol and barbiturates enhance GABA-stimulated influx of ³⁶Cl⁻ in isolated brain membranes. *Pharmacologist* 27:125, 1985.

- Allan, A.M., and Harris, R.A. Gamma-aminobutyric acid and alcohol actions: Neurochemical studies of long sleep and short sleep mice. *Life Sci* 39:2005-2015, 1986.
- Allan, A.M., and Harris, R.A. Involvement of neuronal chloride channels in ethanol intoxication, tolerance, and dependence. In: Galanter, M., ed. *Recent Developments in Alcoholism*. Vol 5. New York: Plenum Press, 1987. pp. 313-325.
- Basile, T.; Hoffer, B.; and Dunwiddie, T. Differential sensitivity of cerebellar Purkinje neurons to ethanol in selectively outbred lines of mice: Maintenance in vitro independent of synaptic transmission. *Brain Res* 264:69-78, 1983.
- Bickford-Wimer, P.; Granholm, A.-C.; Bygdeman, M.; Hoffer, B.J.; Olson, L.; Seiger, Å.; and Strömberg, I. Human fetal cerebellar and cortical tissue transplanted to the anterior eye chamber of athymic rats: Electrophysiologic and structural studies. *Proc Natl Acad Sci USA* 84:5957-5961, 1987.
- Bloom, F.E., and Siggins, R.G. Electrophysiologic action of ethanol at the cellular level. *Alcohol* 4:331-337, 1987.
- Bonetti, E.P.; Burkard, W.P.; Gabl, M.; and Mohler, H. The partial inverse benzodiazepine agonist Ro 15-4513 antagonizes acute ethanol effects in mice and rats. *Br J Pharmacol* 86:463P, 1985.
- Bonetti, E.P.; Polc, P.; and Pieri, L. An azido analogue of the benzodiazepine antagonist Ro 15-1788 (Ro 15-4513) behaves as a partial inverse benzodiazepine agonist. *Neurosci Lett Suppl* 18:S30, 1984.
- Braestrup, C.; Honroe, T.; Nielsen, M.; Petersen, E.N.; and Jensen, L.H. Ligands for benzodiazepine receptors with positive and negative efficacy. *Biochem Pharmacol* 33:859-862, 1984.
- Brodie, M.S.; Lee, K.; Fredholm, B.B.; Ståhle, L.; and Dunwiddie, T.V. Central versus peripheral mediation of responses of adenosine receptor

- agonists: Evidence against a central mode of action. *Brain Res* 415:323-330, 1987.
- Burch, T.P.; and Ticku, M.K. Ethanol enhances [3H]diazepam binding at the benzodiazepine-GABA-receptor-ionophore complex. *Eur J Pharmacol* 67:325-326, 1980.
- Carlen, P.L.; Gurevich, N.; and Durand, D. Ethanol in low doses augments calcium-mediated mechanisms measured intracellularly in hippocampal neurons. *Science* 215:306-309, 1982.
- Celentano, J.J.; Gibbs, T.T.; and Farb, D.H. Ethanol potentiates GABA- and glycine-induced chloride currents in chick spinal cord neurons. *Brain Res* 455:377-380, 1988.
- Cott, J.; Carlsson, A.; Engel, J.; and Lindqvist, M. Suppression of ethanol-induced locomotor stimulation by GABA-like drugs. *Naanyin-Schmiedebergs Arch Pharmacol* 295:203-209, 1974.
- Curtis, D.R., and Felix, D. The effect of bicuculline upon synaptic inhibition in the cerebral and cerebellar cortices of the cat. *Brain Res* 34:301-321, 1971.
- Davidoff, R.A. Alcohol and presynaptic inhibition in an isolated spinal cord preparation. *Arch Neurol* 28:60-63, 1973.
- Dray, A. Comparison of bicuculline methochloride with bicuculline and picrotoxin as antagonists of amino acid and monoamine depression of neurones in the rat brainstem. *Neuropharmacology* 14:887-891, 1975.
- Dunwiddie, T.V.; Basile, A.S.; and Palmer, M.R. Electrophysiological responses to adenosine analogs in rat hippocampus and cerebellum: Evidence for mediation by adenosine receptors of the A₁ subtype. *Life Sci* 34:37-47, 1984.
- Dunwiddie, T.V., and Fredholm, B.B. Adenosine modulation of synaptic responses in rat hippocampus: Possible role of inhibition or activation of adenylate cyclase. In: Cooper, D.M.F., and Seamon, K.B., eds. *Advances*

in Cyclic Nucleotide and Protein Phosphorylation Research. New York: Raven Press, 1985. pp. 259-272.

Festing, M.W.; May, D.; Connors, T.A.; Lovell, D.; and Sparrow, S. An athymic nude mutation in the rat. *Nature* (London) 274:365-366, 1978.

Geller, H.M.; Taylor, D.A.; and Hoffer, B.J. Benzodiazepines and central inhibitory mechanisms. *Naanyn-Schmiedebergs Arch Pharmacol* 304:81-88, 1978.

George, F., and Chu, N.-S. Effects of ethanol on Purkinje cells recorded from cerebellar slices. *Alcohol* 1:353-358, 1984.

Gerhardt, G.A., and Palmer, M.R. Characterization of the techniques of pressure ejection and microiontophoresis using in vivo electrochemistry. *J Neurosci Methods* 22:147-159, 1987.

Granholm, A.-C.; Eriksdotter-Nilsson, M.; Strömberg, I.; Stieg, P.; Seiger, Å.; Olson, L.; Bygdeman, M.; Geffard, M.; Oerthel, W.; Dahl, D.; Hoffer, B.J.; and Freedman, R. Morphological and electrophysiological studies of human hippocampal transplants in the anterior eye chamber of athymic nude rats. *Exp Neurol* 104:162-171, 1989.

Granholm, A.-C.E., and Palmer, M.R. Electrophysiological effects of norepinephrine on Purkinje neurons in intraocular cerebellar grafts: α - vs β -specificity. *Brain Res* 459:256-264, 1988.

Hall, M.E.; Granholm, A.-C.; Stevens, J.O.; and Hoffer, B.J. Myocardium and cortex cereberi xenografts transplanted into the anterior chamber of the eye of athymic rats: A morphologic and electrophysiological profile. *Neurosci Lett* 75:89-94, 1987.

Harris, D.P., and Sinclair, J.G. Ethanol-GABA interactions at the rat Purkinje cell. *Gen Pharmacol* 15:449-454, 1984.

Harris, R.A.; Allan, A.M.; Daniell, L.C.; and Nixon, C. Antagonism of ethanol and pentobarbital actions by benzodiazepine inverse agonists: Neurochemical studies. *J Pharmacol Exp Ther* 247:1012-1017, 1988.

- Hoffman, P.L.; Tabakoff, B.; Szabo, G.; Suzdak, P.D.; and Paul, S.M. Effect of imidazobenzodiazepine, Ro 15-4513, on the incoordination and hypothermia produced by ethanol and pentobarbital. *Life Sci* 41:611-619, 1987.
- Liljequist, S., and Engel, J. Effects of GABAergic agonists and antagonists on various ethanol-induced behavioral changes. *Psychopharmacology* 78:71-75, 1982.
- Lister, G.R., and Nutt, D.J. Is Ro 15-4513 a specific alcohol antagonist? *Trends Neurosci* 10:223-225, 1987.
- Martz, A.; Deitrich, R.A.; and Harris, R.A. Behavioral evidence for the involvement of γ -aminobutyric acid in the actions of ethanol. *Eur J Pharmacol* 89:53-62, 1983.
- Mehta, A.K., and Ticku, M.K. Ethanol potentiation of GABAergic transmission in cultured spinal cord neurons involves γ -aminobutyric acid-gated chloride channels. *J Pharmacol Exp Ther* 246:558-564, 1988.
- Mereu, G., and Gessa, G.L. Low doses of ethanol inhibit the firing of neurons in the substantia nigra, pars reticulata: A GABAergic effect? *Brain Res* 360:325-330, 1985.
- Nestoros, J.N. Ethanol specifically potentiates GABA-mediated neurotransmission in feline cerebral cortex. *Science* 209:708-710, 1980.
- Nutt, D.J., and Lister, R.G. The effect of the imidazodiazepine Ro 15-4513 on the anticonvulsant effects of diazepam, sodium pentobarbital and ethanol. *Brain Res* 413:193-196, 1987.
- Olson, L.; Seiger, Å.; and Strömberg, I. Intraocular transplantation in rodents. A detailed account of the procedure and examples of its use in neurobiology with special reference to brain tissue grafting. In: Federoff, S., and Hertz, eds. *Advances in Cellular Neurobiology*. Vol. 4. New York: Academic Press, 1983. pp. 407-422.

- Olson, L.; Strömberg, I.; Bygdeman, M.; Granholm, A.-C.; Hoffer, B.J.; Freedman, R.; and Sieger, Å. Human fetal tissue grafted to rodent hosts: Structural and functional observations of brain, adrenal and heart tissue in oculo. *Exp Brain Res* 67:163-178, 1987.
- Palmer, M.R. Micro pressure-ejection: A complementary technique to microiontophoresis for neuropharmacological studies in the mammalian central nervous system. *J Electrophysiol Tech* 9:123-139, 1982.
- Palmer, M.R.; Fossum, L.H.; and Gerhardt, G.A. Micro pressure-ejection techniques for mammalian neuropharmacological investigations. In: Geller, H.M., ed. *Electrophysiological Techniques in Pharmacology*, New York: Alan R. Liss, 1986. pp. 169-187.
- Palmer, M.R., and Hoffer, B.J. Catecholamine modulation of enkephalin-induced electrophysiological responses in cerebral cortex. *J Pharmacol Exp Ther* 213:205-215, 1980.
- Palmer, M.R.; Sorensen, S.R.; Freedman, R.; Olson, L.; Hoffer, B.J.; and Seiger, Å. Differential ethanol sensitivity of intraocular cerebellar grafts in long-sleep and short-sleep mice. *J Pharmacol Exp Ther* 222:480-487, 1982.
- Palmer, M.R.; van Horne, C.G.; Harlan, T.J.; and Moore, E.A. Antagonism of ethanol effects on cerebellar Purkinje neurons by the benzodiazepine inverse agonists Ro 15-4513 and FG 7142: Electrophysiological studies. *J Pharmacol Exp Ther* 247:1018-1024, 1988.
- Phillis, J.W., and Tebecis, A.K. The responses of thalamic neurones to iontophoretically applied monoamines. *J Physiol* 192:715-745, 1967.
- Polc, P. Interactions of partial inverse benzodiazepine agonists Ro 15-4513 and FG 7142 with ethanol in rats and cats. *Br J Pharmacol* 86:465P, 1985.
- Pruett, J.K.; Walle, T.; and Walle, U.K. Propranolol effects on membrane repolarization time in isolated canine Purkinje fibers: Threshold tissue content and the influence of exposure time. *J Pharmacol Exp Ther* 215:539-543, 1980.

- Rogers, J.; Siggins, G.R.; Schulman, J.A.; and Bloom, F.E. Physiological correlates of ethanol intoxication, tolerance and dependence in rat cerebellar Purkinje cells. *Brain Res* 196:183-198, 1980.
- Seil, F.J.; Leiman, A.L.; Herman, M.M.; and Fisk, R.A. Direct effects of ethanol on central nervous system cultures: An electrophysiological and morphological study. *Exp Neurol* 55:390-404, 1977.
- Siggins, G.R.; Pittman, Q.J.; and French, E.D. Effects of ethanol on CA1 and CA3 pyramidal cells in the hippocampal slice preparation: An intracellular study. *Brain Res* 414:22-34, 1987.
- Sinclair, J.G., and Lo, G.F. The effect of ethanol on cerebellar Purkinje cell discharge pattern and inhibition evoked by local surface stimulation. *Brain Res* 204:465-471, 1981.
- Skolnick, P., and Paul, S.M. Benzodiazepine receptors. *Annu Rep Med Chem* 16:21-29, 1981.
- Sorensen, S.; Carter, D.; Marwaha, J.; Baker, R.; and Freedman, R. Disinhibition of cerebellar Purkinje neurons from noradrenergic inhibition during the rising phase blood ethanol. *J Studies Alcohol* 42:908-917, 1981.
- Sorensen, S.; Palmer, M.; Dunwiddie, T.; and Hoffer, T. Electrophysiological correlates of ethanol-induced sedation in differentially sensitive lines of mice. *Science* 210:1143-1145, 1980.
- Stone, T.W. *Microiontophoresis and Pressure Ejection*. New York: John Wiley & Sons, 1985.
- Straughan, D.W., and Watson, J.F. Monoamine effects on cortical neurones and γ -aminobutyric acid. In: *5th International Congress on Pharmacology*, 1972. p. 223.
- Suzdak, P.D.; Glowa, J.R.; Crawley, J.N.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. A selective imidazobenzodiazepine antagonist of ethanol in the rat. *Science* 234:1243-1247, 1986a.

Suzdak, P.D.; Schwartz, R.D.; Skolnick, P.; and Paul, S.M. Ethanol stimulates gamma-aminobutyric acid receptor mediated chloride transport in rat brain synaptoneuroosomes. *Proc Natl Acad Sci USA* 83:4071-4075, 1986b.

Ticku, M.K.; Lowrimore, P.; and Lehoullier, P. Ethanol enhances GABA-induced ^{36}Cl influx in primary spinal cord cultured neurons. *Brain Res Bull* 17:123-126, 1986.

Vos, J.G.; Kreeftenberg, J.G.; Kruijt, B.C.; Kruizinga, W.; and Steerenberg, P. The athymic nude rat. II. Immunological characteristics. *Clin Immunol Immunopathol* 15:229-237, 1980.

Woodward, D.J.; Hoffer, B.J.; Siggins, G.R.; and Bloom, F.E. The ontogenetic development of synaptic junctions. Synaptic activation and responsiveness to neurotransmitter substances in rat cerebellar Purkinje cells. *Brain Res* 34:73-97, 1971.



<http://nihlibrary.nih.gov>

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080



NIH LIBRARY



3 1496 01022 8107